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### CORRECTIONS

On page 199, Vol. 123, No. 1, March, 1938, line 3 from the foot of the page, read *3.2 to 3.4 gm.* for *13.2 to 13.4 gm.*

On page 201, line 8, read *0.2* for *0.4*.

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# GLYCOLYSIS AND GLUTATHIONE

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WITH THE TECHNICAL ASSISTANCE OF B. WAGNER

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Dudley (2), who discovered the inhibitory action of moniodo acid on the mutase enzymatic system responsible for the conversion of methylglyoxal into lactic acid, thought that this might explain the inhibitory effect of halogenated acids upon the glycolytic process. Lohmann (4), however, found that reduced glutathione is the coenzyme of methylglyoxalase. Later Lohmann (5) also showed that the inhibition of lactic acid formation by 0.01 M moniodoacetic acid was due to destruction of the reduced glutathione, the coenzyme of the methylglyoxalase. Dickens (1) likewise observed the inhibition of glyoxalase by moniodoacetic acid and the complete restoration of its activity by the addition of reduced glutathione. Yamazoye (15) using a filtered liver autolysate with hexosediphosphate found that in the absence of reduced glutathione no lactic acid was formed, while methylglyoxal accumulated. In the presence of glutathione there was a considerable production of lactic acid but the other enzymes were not affected, since the reduced glutathione acts only upon the glyoxalase.

It seemed desirable to study the relationship between the reduced glutathione content and the glycolytic activity of blood, although from Lohmann's (4) experiments it appears that some well glycolyzing muscle extracts may be —SH-free. An examination of various bloods has shown that, although there may be an apparent relationship between the reduced glutathione and the glycolytic power, it does not bear closer scrutiny. Neither in rabbit, dog, nor in sheep blood could any correlation be found between the glycolytic activity and the reduced glutathione con-

tent. Only in hog blood, which is a very poorly glycolyzing system and has a very low GSH content, was the average GSH for glycolyzing bloods appreciably higher than for non-glycolyzing bloods (16.9 and 11.2 mg. per cent). However, there was so much overlapping in the GSH content of various hog bloods that no correlation between GSH concentration and glycolysis could be established. Furthermore, even doubling the glutathione content by the addition of crystalline reduced glutathione to blood in no way altered its glycolytic capacity. The sugar curves with or without added reduced glutathione were identical. The added reduced glutathione rapidly disappears, so that in about 2 hours practically no difference between the original and the treated blood can be found.

Although our study failed to establish a relationship between the reduced glutathione content of blood and its glycolytic activity, certain facts pertaining to the changes in the reduced glutathione concomitant with glycolysis deserve to be recorded.

### *Methods*

Blood was collected as nearly aseptically as possible. Whenever the bloods were secured outside the laboratory, the container was immediately placed on ice and the blood was worked over as soon as it was brought to the laboratory. Except in a few instances, the blood was defibrinated by shaking gently with glass beads. Samples for analysis were taken immediately and again at definite intervals during the incubation of the blood at 38°. Sugar was determined on the Somogyi (11) filtrate by the Shaffer-Somogyi (10) procedure. We found that true sugar values are obtained by this combination. The reduced glutathione was determined on a sulfosalicylic acid filtrate by the iodate titration method of Okuda and Ogawa (9), the titration being carried out in a cooling mixture.

In the early experiments the total glutathione was also determined, after preliminary reduction by zinc powder. Nothing significant was found from these determinations, since in every instance where this was done the total glutathione did not change, and the disappearance of GSH could be accounted for by an oxidation to the GS—SG state. In later experiments only the reduced glutathione was determined.

## EXPERIMENTAL

*Hog Blood*—Hog blood is generally considered to be non-glycolyzing but in a large series of determinations we found that only approximately 60 per cent of the blood samples do not glycolyze, the other 40 per cent showing a variable but moderate degree of glycolysis. In oxalated pig blood the reduced glutathione invariably decreases during incubation, about 21 per cent in 2 to 3 hours. The determination of total glutathione showed that there was an actual oxidation of the GSH to the GS—SG form. For reasons which are not clear, oxalating of the blood leads to a marked decrease in the reduced glutathione on incubation. We observed the same in the case of dog bloods, though not to the same extent as in hog blood. Of six oxalated hog bloods, in one experiment, two did not glycolyze, and showed an average decrease in GSH by 5.2 mg. per cent (35 per cent). In the remaining glycolyzing bloods the GSH decreased only about 14 per cent. In other words, the GSH was oxidized to GS—SG very much less in glycolyzing than in non-glycolyzing bloods. The results with defibrinated blood, though not so marked quantitatively, were even more impressive. These experiments have shown that during  $2\frac{1}{2}$  to 3 hours of incubation the GSH of the non-glycolyzing hog bloods decreased by 0.6 to 5.6 mg. per cent, but in the glycolyzing bloods the change in GSH was either entirely within the experimental error or the GSH actually increased in the early stages.

*Dog Blood*—In a series of experiments with oxalated dog blood we found a definite decrease of 10 to 20 per cent in the GSH. In another series three samples of defibrinated blood were used, the rate of glycolysis and the changes in GSH being recorded graphically in Fig. 1. In two samples the GSH increases markedly during the 1st hour but remains unchanged in the 3rd. Following this, and when about two-thirds of the sugar has already disappeared, the GSH curves begin to decline very markedly and at an increasing rate as the glycolysis is being completed. After 6 hours the GSH has decreased by one-half or more.

*Rabbit Blood*—In Fig. 1 are also shown the results obtained with three different samples of defibrinated rabbit blood in which the glycolysis was completed within 5 to 6 hours. A glance at the GSH curves reveals a rather remarkable constancy and, even at the completion of the glycolysis, the GSH content in only one of

the bloods showed a relatively small decrease. Occasionally a rabbit blood showed an initial rise in the GSH during glycolysis. When the time of glycolysis was prolonged by adding 200 mg. per cent of extra glucose, it was found that even after 11 hours, while only about half of the sugar glycolyzed, the GSH was still practically unchanged.

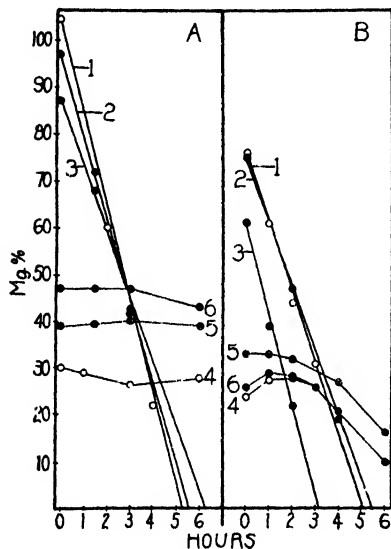


FIG. 1

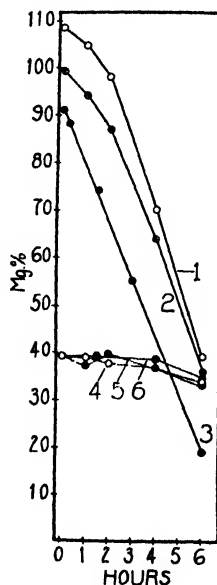


FIG. 2

FIG. 1. A, glycolysis of rabbit blood; B, glycolysis of dog blood.

Curves 1, 2, and 3 represent glucose (glycolysis curve); Curves 4, 5, and 6, reduced glutathione (GSH).

FIG. 2. Glycolysis of rabbit blood on 3 consecutive days. Curves 1, 2, and 3 represent glucose; Curves 4, 5, and 6, GSH.

However, if one follows the GSH curve several hours after the completion of glycolysis, the GSH content of the rabbit blood is found to decrease substantially. Thus, in four different bloods in which the GSH did not vary by more than 1 to 2 mg. per cent during the entire period of glycolysis, the GSH content decreased very much in 2 or 3 hours after glycolysis (5 to 13 mg. per cent, or 17 to 33 per cent of the initial concentration).

In this connection we wish to describe an experiment with rabbit

blood which presents some interesting points, the results of which are shown graphically in Fig. 2. Usually the glycolytic process follows a linear course, indicating that the reaction probably is confined to the surface of the cells. We came across a blood which showed an abnormal behavior in that the curve of glycolysis was not linear. The rate for the first 2 or 3 hours was unusually slow but increased afterwards and became linear. This blood was tested on 3 consecutive days, during which it was preserved in the ice box. The sugar concentration decreased from day to day, showing that glycolysis was not entirely checked by the cold. On the 2nd day the deviation from the linear course of the glycolysis curve was less pronounced and a glycolysis experiment performed on the 3rd day yielded a typical linear curve from the start. This blood was a decidedly slow glycolyzing agent, requiring between 8 and 9 hours instead of the usual 5 to 6 hours for the completion of the process. The reduced glutathione concentration of this blood did not change during the 3 days while it was preserved in the ice box, and the GSH curves during the glycolysis experiments are remarkably similar, showing a definite decrease only during the advanced stages of the process.

*Effect of Inhibiting Respiration*—To determine whether acceleration or inhibition of glycolysis affects the reduced glutathione, the following experiment was performed with defibrinated rabbit blood. The sample was divided into three portions; one (control) was diluted with  $M/8$  NaCl, the second with  $M/8$  NaF, and the third with  $M/8$  NaCl containing KCN. The fluoride practically completely suppressed the glycolysis, but the sample poisoned with cyanide showed a great increase in the rate of glycolysis. If the rate between the 6th and 12th hour of glycolysis were maintained unchanged, the sugar in the control sample would disappear completely in  $21\frac{1}{2}$  hours, that in the cyanide sample in 15 hours; in other words, the latter glycolyzed 1.5 times as fast as the former. The GSH curves of the control and of the cyanide bloods were indistinguishable from each other (Fig. 3), but in the fluoride blood the reduced glutathione began to decline at once, losing about 60 per cent in 12 hours.

In a second experiment defibrinated rabbit blood was diluted with an equal volume of  $M/8$  NaCl or of  $M/8$  NaF containing glucose. In this experiment the GSH curves were followed for 20 hours



(Fig. 4). In the whole blood, as well as in the blood diluted with NaCl, the GSH curves run a remarkably parallel course. The behavior of the GSH in the non-glycolyzing, fluoride-poisoned

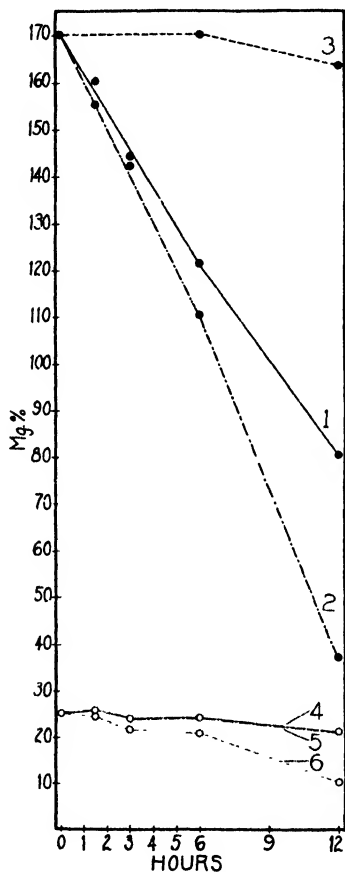


FIG. 3. Glycolysis of rabbit blood diluted 3:1 with m/8 NaCl, control (Curve 1 glucose, Curve 4 GSH), with m/8 NaCl containing  $1 \times 10^{-3}$  N KCN (Curve 2 glucose, Curve 5 GSH), and with m/8 NaF (Curve 3 glucose, Curve 6 GSH).

blood is interesting because in this case the initial rise is missing and the GSH remains practically unchanged for 2 hours, then decreases progressively and much more rapidly than in the other

two samples. At the end of 20 hours nearly 90 per cent of the GSH had disappeared.

These experiments show that there is considerable residual glycolytic capacity (at least 50 per cent) in rabbit blood which can be realized by suppressing its respiratory activity with KCN without affecting the changes in GSH. Suppressing glycolysis, however, by means of fluoride leads to an almost immediate decrease in the

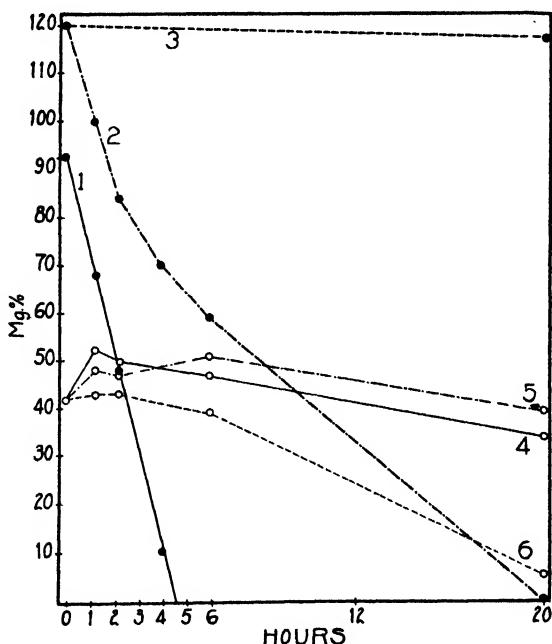


FIG. 4. Glycolysis of whole rabbit blood (Curve 1 glucose, Curve 4 GSH), and of blood diluted with 1:1 m/8 NaCl (Curve 2 glucose, Curve 5 GSH) or with m/8 NaF (Curve 3 glucose, Curve 6 GSH).

GSH, which becomes gradually more rapid until the GSH disappears practically entirely. This illustrates, therefore, once more that the glycolytic process is associated with oxidation-reduction reactions which prevent the oxidation of the GSH to the GS-SG.

*Experiments with Washed Blood Cells*—The previous experiments indicate that although different types of blood show variations in detail, the level of the reduced glutathione in all of them tends to

be maintained or even to be raised while glycolysis is proceeding, but decreases with greater or less speed when glycolysis has been completed or suppressed. Experiments were made with dog blood cells washed with Tyrode's solution both with and without glucose. The sugar and GSH were determined initially and at the end of 2 to 3 hours of incubation. The results are given in Table I.

This experiment shows strikingly that in glycolyzing cells the GSH is maintained for 2 or 3 hours practically unchanged, but where no glycolysis is taking place, the GSH decreases or disappears.

These experiments again bring out the essential and striking fact that glycolysis is directly associated with maintaining the level of the reduced glutathione of the blood. In other words, during glycolysis agencies are operative which furnish the reduc-

TABLE I  
*Glycolysis of Washed Red Blood Cells of Dog*

Time	Glycolysis	Glutathione		
		Initial	Final	Difference
<i>hrs.</i>	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
2	50	5.4	5.4	0
2	0	5.5	0	-5.5
{2	29	20.2	20.6	+0.4
{3	50	20.2	20.2	0
3	0	18.3	14.9	-3.4

tion potential necessary to prevent the oxidation of GSH to GS-SG.

Different mammalian bloods manifest a different behavior. Thus, in dog blood the GSH begins to decrease before glycolysis has been completed, the decrease proceeding very rapidly. Rabbit blood differs from this in that, generally, the GSH is maintained more or less constant, or certainly does not suffer any appreciable loss, until after the glycolytic process has run its full course. Similar results were obtained with glycolyzing hog bloods, while in the non-glycolyzing hog bloods the GSH showed a decided decrease during the same length of time. When the glycolysis of rabbit blood is suppressed by means of fluoride, the decrease in GSH commences almost immediately and progresses much further than in the glycolyzing control bloods.

Hopkins and Elliott (3) found that the behavior of the GSH is very different in liver tissue from well fed and from fasted animals. In the former, the GSH may remain constant or even rise for some time before a progressive fall sets in, leading ultimately to complete oxidation of the —SH group. In liver tissue from fasting animals, however, this preliminary phase is either much shortened or does not exist at all. Of course, in a fasting animal the liver glycogen is more or less rapidly exhausted and these experiments illustrate that the glucose content of the tissue suspension is very essential in furnishing the reducing agencies necessary for maintaining the GSH level observed during the first 2 hours of incubation. That this depends upon some enzymatic mechanism (glycolysis?) is further substantiated by the observation of these authors that heating the liver tissue to 50° under anaerobic conditions destroys its ability to keep the GSH in the reduced condition on aeration. In this case the GSH not only begins to disappear immediately but the loss is also more rapid. Oberst (8), studying the disappearance of GSH from human blood, reports an experiment with whole blood and with hemolyzed blood. At the end of 5 hours (see Table I, p. 11 (8)) the GSH of the whole blood has somewhat increased (1.7 mg. per cent) while that of the hemolyzed blood had decreased 23 mg. per cent. The relation of the changes in GSH to glycolysis in this experiment is clearly obvious, since glycolysis is entirely lacking in hemolyzed blood, and this experiment thus substantiates our main thesis.

We noted that the GSH sometimes increases during the first 2 hours of incubation. This initial rise is much more common in dog than in rabbit blood, and we also observed this in human blood. This phenomenon had already been described by Hopkins and Elliott (3) in the paper referred to on the behavior of the GSH of liver tissue suspensions and also by Meldrum (6), who demonstrated the reduction of glutathione by isolated mammalian red blood cells. Meldrum concluded from his studies that glucose alone cannot reduce the disulfide glutathione, the reduction being a function of the intact blood corpuscles. We are further in agreement with his conclusion that this reduction of GS — SG to GSH is probably not affected by glycolysis.

*Effect of Arsenate*—Since it was shown that arsenate stimulates glycolysis of rabbit blood (7), a series of experiments was carried

out to determine its effect on the reduced glutathione. In one experiment (Fig. 5) defibrinated rabbit blood was diluted (3:1) with Locke's solution or with Locke's solution containing enough  $\text{Na}_2\text{HAsO}_4$  to give a final concentration of  $\text{m}/150$  and  $\text{m}/300$ . During the first 4 hours there was considerable stimulation of glycolysis but afterwards a marked inhibition occurred. While the glycolysis of the control sample was completed in a little over 7 hours,

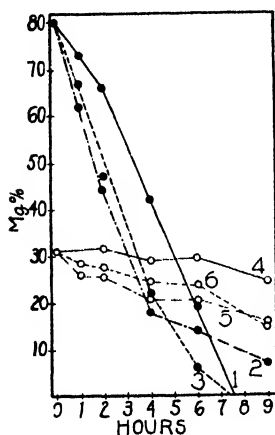


FIG. 5

FIG. 5. Glycolysis of rabbit blood, untreated, control (Curve 1 glucose, Curve 4 GSH), and treated with  $\text{m}/150$  (Curve 2 glucose, Curve 5 GSH) and  $\text{m}/300$  sodium arsenate (Curve 3 glucose, Curve 6 GSH). All three bloods were diluted 3:1 with isotonic salt solution.

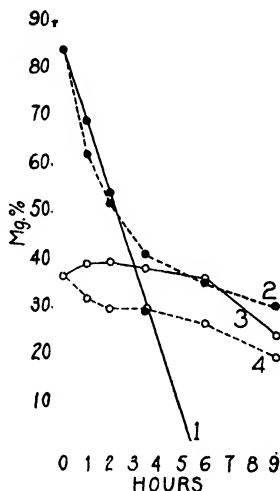


FIG. 6

FIG. 6. Glycolysis of rabbit blood, untreated, control (Curve 1 glucose, Curve 3 GSH), and treated with 0.01  $\text{m}$  sodium arsenate (Curve 2 glucose, Curve 4 GSH).

the glycolysis in  $\text{m}/150 \text{ Na}_2\text{HAsO}_4$  was not quite complete even after 9 hours.

In a previous study, Morgulis and Pinto (7) have shown that arsenate, which inhibits glycolysis of dog blood even in very small concentration, stimulates glycolysis of rabbit blood. This was based on a study of glycolysis for only 2 to 4 hours. The inhibition does not generally manifest itself until after 3 to 4 hours, though it may appear even earlier with strong concentrations. As will

be seen from this and subsequent experiments, the effect of arsenate may be divided into two phases, an early phase of *stimulation* and a later phase of *inhibition*, the degree of each depending upon the arsenate concentration. The behavior of the GSH was also affected in a striking manner by the arsenate. Whereas in this experiment (Fig. 5) the GSH of the untreated blood varied only slightly during the first 6 hours, and dropped abruptly in the next 3 hours, in the arsenate bloods a marked decrease in the GSH took place after 1 hour of glycolysis. The decrease was more or less continuous and was greater in the M/150 than in the M/300 arsenate. Similar results were obtained in other experiments with arsenate concentrations ranging from M/300 to M/900.

To avoid the factor of dilution, glycolysis experiments were also carried out in which the necessary amount of  $\text{Na}_2\text{HAsO}_4$  was dissolved directly in the blood. In Fig. 6 the results of an experiment are plotted in which the effect of 0.01 M  $\text{Na}_2\text{HAsO}_4$  was studied. The glycolysis and the GSH curves of the control conform to what has been said previously. But in the arsenate blood, following a brief stimulation which lasted about an hour, glycolysis becomes progressively inhibited, so that after 9 hours only about 65 per cent of the sugar has disappeared. The behavior of the GSH, however, is markedly different, decreasing 5 mg. per cent in the 1st hour and 7.5 mg. per cent after 4 hours. The inhibition of glycolysis at this point becomes very pronounced and the GSH falls rapidly.

In Fig. 7 the results of an experiment with M/150 and M/300 arsenate are plotted. The glycolysis in the arsenate sample is at first greatly increased and exceeds the control by 30 and 40 per cent, respectively, but after 3 hours the glycolysis becomes definitely inhibited. The GSH of the arsenate-treated bloods again shows an abrupt fall from the start, decreasing 8 and 11.5 mg. per cent in 3 hours. The subsequent loss in GSH runs more or less parallel to that of the control blood.

In Fig. 8 the results of an experiment with M/300 and M/600 arsenate are recorded. The glycolysis in the M/300 sample is greatly stimulated but after the 2nd hour the onset of inhibition becomes apparent and the rate slows down progressively, so that the completion of the process is much delayed. The initial stimulation in the M/600 sample is not so great but no inhibition develops and the rate of glycolysis is about 55 per cent greater than in the control.

On comparison of the glycolysis of the  $m/600$  arsenate sample with that of a previous experiment (Fig. 3) it may be stated that the rate of glycolysis has been stimulated to the same extent as by  $0.001$   $M$  KCN. The GSH curves of the arsenate-treated blood, though running parallel to the control, do not show the initial rise and show a continuous decrease, which is greater in the higher concentration.

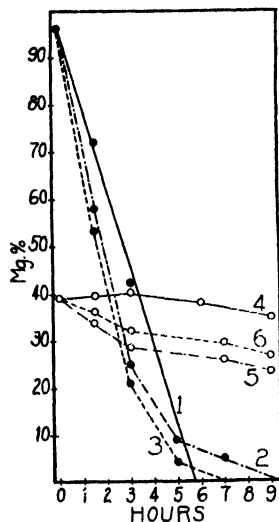


FIG. 7

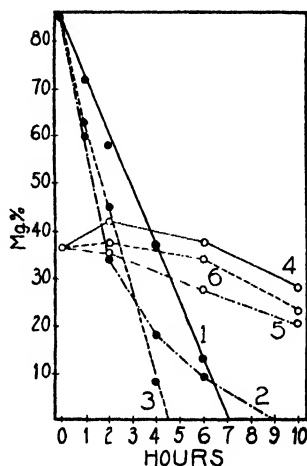


FIG. 8

FIG. 7. Glycolysis of rabbit blood, untreated, control (Curve 1 glucose, Curve 4 GSH), and treated with  $m/150$  (Curve 2 glucose, Curve 5 GSH) and with  $m/300$  sodium arsenate (Curve 3 glucose, Curve 6 GSH).

FIG. 8. Glycolysis of rabbit blood, untreated, control (Curve 1 glucose, Curve 4 GSH), and treated with  $m/300$  (Curve 2 glucose, Curve 5 GSH) and with  $m/600$  sodium arsenate (Curve 3 glucose, Curve 6 GSH).

From the results on the effect of arsenate ( $0.01$   $M$  to  $m/900$ ) it is obvious that the stimulating effect upon glycolysis lasts longer the smaller the concentration and, depending upon the concentration, this is followed by a greater or less inhibition. The initial stimulating effect on glycolysis is apparently associated with an inhibition of the respiratory function. The arsenate-treated bloods retain a definitely brighter hue than the control bloods in which the oxy-hemoglobin becomes partially reduced. The behavior of the GSH in

the arsenate experiments, however, is surprisingly affected. It decreases from the very start, whereas in the controls it may remain fairly constant (or even show an initial rise) during the entire glycolytic process. The total decrease in GSH is invariably much greater than in the controls and, furthermore, varies with the concentration of  $\text{Na}_2\text{HAsO}_4$ , although the difference depends entirely on the loss of GSH during the first 2 hours of the experiment.

The effect of arsenate on rabbit blood expresses itself thus: first, in both stimulating and inhibiting its glycolysis and, second, in a more or less rapid loss of reduced glutathione. The inhibitory phase of the arsenate effect was overlooked before (7), because our study was limited to a 2 to 4 hour incubation period and we did not follow the entire glycolysis curve, but merely determined the sugar at the beginning and end of an experiment. In this way, even where inhibition had already taken place, the determination of a single point in the course of glycolysis failed to make this manifest. The effect of arsenate on the entire course of glycolysis manifests itself in an initial stimulation alternating with an inhibition which is greater the higher the arsenate concentration. The duration of the stimulation phase seems to be generally shorter the higher the concentration. Following the initial stimulation, the inhibition produced by 0.01 M or M/150  $\text{Na}_2\text{HAsO}_4$  may be very great. In our previous paper it was pointed out that the effect of arsenate on glycolysis of dog blood was entirely different, inhibition being produced even by very small concentrations. The results of the present experiments, showing that in rabbit blood an inhibition proportional to the arsenate concentration likewise follows the initial stimulation and that the GSH shows a more or less extensive loss from the start, seemed therefore perplexing. The only explanation that appears reasonable is that arsenate exerts an inhibitory effect on glycolysis. Indeed, Tanzi (13) studying the glycolysis of the isolated rabbit heart finds that this is inhibited by 0.002 M  $\text{Na}_2\text{HAsO}_4$ , and this fits in with our earlier finding that the glycolysis of dog blood is inhibited.

But, if arsenate inhibits glycolysis, how are we to account for the initial stimulation observed in the case of rabbit blood? Tanzi (13) states that the glycolysis of the rabbit heart is stimulated by sodium arsenite. This is probably not correct. Arsenite, as was shown by Szent-Györgyi (12), has no effect on



fermentation but inhibits very strongly cellular respiration, causing 25 per cent inhibition in oxygen uptake of liver tissue even in a concentration of 0.00004 M. Voegtlin and his collaborators (14) have shown that arsenite, but not arsenate, strongly inhibits cellular respiration. The initial stimulation of glycolysis may, thus, be regarded as resulting from suppression of the respiratory activity, as was also found to be the case when KCN acts on rabbit blood. Although we used an arsenate of very high purity, the arsenate salts actually contain a few thousandths of 1 per cent of arsenite, and this would suffice to account for the initial increase in glycolysis through the suppression of respiration.

In this event the observed stimulation of glycolysis is really the resultant of two opposed phenomena: the (*direct*) inhibition by arsenate and the (*indirect*) stimulation by arsenite, the latter exerting its action in much smaller concentrations than the former. This will explain why with a low concentration of arsenate (M/600) the glycolysis was completed in about two-thirds of the time required by the control without any inhibition being manifested, while with a strong concentration (0.01 M) the stimulation is much less in extent and duration, and is followed by a strong inhibition.

If this view is correct, the question may naturally be asked, how this would explain the two phase effect of the arsenate. If the arsenite represses the respiratory function and thus promotes the glycolysis (Pasteur's reaction), then why does the stimulation of glycolysis give place somewhat abruptly to an inhibition? The arsenite, which poisons respiration but does not influence fermentation, is effective in a very much smaller concentration than arsenate, which inhibits glycolysis but does not affect respiration. In this connection the behavior of the reduced glutathione becomes intelligible. We have already pointed out that, contrary to all our experience with glycolyzing rabbit bloods, the GSH in the arsenate-treated bloods invariably decreases, the decrease varying with the concentration. This, at first sight, paradoxical behavior of the GSH in the arsenate bloods is just what might be expected on the assumption that arsenite, invariably present as an impurity in the arsenate, causes indirectly an increased glycolysis by suppressing the respiration. Voegtlin and his collaborators (14) have shown that reduced glutathione paralyzes the action of

arsenite upon cellular respiration. They suggested that possibly an inactive compound is formed; at any rate, the inhibitory action of arsenite can be overcome entirely, when the molar concentrations of GSH and arsenite are in a ratio of about 10:1. It will be noted from the experiments presented in Figs. 5 to 8 that the loss of GSH, which begins immediately, attains its maximum at about the time when the glycolysis curves show the sharp inflection marking the transition from the stimulatory to the inhibitory phase. Although the GSH of the arsenate-treated bloods is decreased much more than that of the control bloods at the close of the experiment, actually the principal loss is occasioned within the first few hours, after which the GSH curves of the treated and untreated bloods run a more or less parallel course. Obviously, the disappearance of the GSH has to do with the removal of the arsenite, this reaction requiring time. But once the arsenite effect has been paralyzed, the purely inhibitory effect of arsenate on glycolysis asserts itself more or less prominently.

#### SUMMARY

During glycolysis oxidation-reduction conditions exist in blood which contribute to maintain the level of the reduced glutathione (GSH). In the absence of glycolysis, the GSH tends to disappear with greater or less speed, becoming oxidized to the disulfide form (GS—SG). In the early phases of the process there may actually be a rise in the GSH, but apparently this is not directly associated with glycolysis. Dog and rabbit blood represent distinct types in this respect, in that in dog blood the GSH begins to disappear even before the glycolytic process has been completed and disappears very rapidly, while in rabbit blood the GSH level, with minor fluctuations, tends to remain practically constant during the entire glycolytic process, disappearing at a moderate rate only after the completion of glycolysis. This behavior of the GSH in relation to glycolysis is demonstrated also with blood cell suspensions, with or without glucose. In the former, the GSH is practically unchanged, whereas in the latter, where no glycolysis is taking place, it tends to disappear. Likewise, suppressing glycolysis with fluoride results in an immediate initiation of the loss of GSH. Stimulation of glycolysis with cyanide had no effect on the glutathione behavior.

A series of experiments was made on rabbit blood treated with arsenate. It was found that in these experiments two sharply defined phases, a stimulatory and an inhibitory phase, appear in the glycolytic process and that the GSH during the first 2 hours undergoes a marked decrease, which varies with the arsenate concentration. The effect of arsenate and of arsenite on the respiratory and glycolytic activity of the blood is discussed, the apparently paradoxical results of the arsenate experiments being interpreted in the light of these different effects.

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# **STUDIES ON SURFACE DENATURATION OF EGG ALBUMIN\***

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**WITH THE TECHNICAL ASSISTANCE OF ALVIN BERMAN**

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Bull and Neurath (1) have reported a study of the kinetics of surface denaturation of egg albumin. The protein solutions were shaken in bottles for varying lengths of time, and the amount of denaturation determined. It was not possible, however, in their studies to establish the effect of the extent and rate of formation of surface. The present paper reports a study of surface denaturation occurring on a drum of known dimensions dipping into a protein solution and rotated at known speeds. This allows one to establish the effect of the rate of creation of new surface and to calculate the amount of denatured protein on unit area of the rotating drum as a function of speed of rotation and of concentration. The principal purpose of this study is thus to extend the work of Bull and Neurath on the kinetics of surface denaturation. It has been found, however, that the amount of denaturation is a smooth function of the speed of rotation of the drum and approaches certain limiting values as the speed of rotation is diminished. The amount of denatured protein in the surface film can thus be extrapolated to zero speed of rotation and an approximation of the conditions and structure of the surface film on a quiescent protein solution is obtained.

## **EXPERIMENTAL**

Investigation was made of three preparations of egg albumin. The results from the first two preparations have been discarded,

\* Presented at the Fourteenth National Colloid Symposium, Minneapolis, 1937.

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since they were used in the development of technique. The egg albumin was prepared from fresh hen's eggs by the method of Kekwick and Cannan (2) and recrystallized four times. It was dialyzed, then electro-dialyzed, and analyzed.

The preparation reported on in this paper had the following characteristics.

pH.....	4.61
Specific conductivity, <i>mhos</i> .....	$2.92 \times 10^{-5}$
Total protein concentration, <i>per cent</i> .....	3.56
Protein, heat-denaturable, <i>per cent</i> .....	93.00
Concentration of heat-denaturable protein, <i>per cent</i> ..	3.31

The preparations were water-clear. The concentration of the protein was taken as the concentration of the heat-denaturable protein.

The protein solutions were preserved by the addition of a small amount of an equal mixture of petroleum oil and washed toluene. They were stored at 2° when not in use.

An ordinary porcelain rotator of the kind used in offices to dampen adhesive surfaces was employed to produce a layer of protein solution whose exact area and rate of formation were known. The rotating drum had the following dimensions, diameter 7.41 cm., circumference 23.4 cm., width 4.95 cm. When the drum was placed in position on its porcelain container, and the standard quantity of protein solution added to the container and rotated, it was found that part of the two sides of the drum was wetted by the solution, and in order to obtain the total surface area wet by rotating the drum, this area had to be added to the area of the face of the drum. This gave a total area of wet surface produced by each rotation of the drum of 171.6 sq. cm.

The drum was rotated by means of a wooden wheel pressed against its edge. The wooden wheel was turned by a strong motor with constant speed through reducing gears. The speed of rotation of the drum was checked many times and found to be constant. The reducing gears allowed speeds of 14.5, 26, 33.5, and 53 revolutions per minute to be used. The wooden wheel was saturated with water before each experiment, in order that none of the solution would be absorbed by it.

The technique of the actual experiment was as follows: A small amount of cotton was placed in the container to remove the sur-

face-denaturated protein from the drum as it rotated. 35 cc. of the desired strength of protein solution were added. The rotator was set in place and the whole apparatus placed on a large balance sensitive to 50 mg., and weighed. The drum was rotated for the desired time and speed, and the container and rotator again placed on the balance, and enough water added to restore the water lost through evaporation. The solution was thoroughly stirred and a portion withdrawn, and its index of refraction determined with a Zeiss dipping refractometer. In the same water bath was a sample of pure water, and the index of refraction of water was subtracted from that of the protein solution. Sufficient time was allowed for the protein solution and water to come to the same temperature, and a number of readings of the index of refraction taken and averaged. In the experiments reported on in this paper, the time of experiment was 5 minutes for the more concentrated and 10 or 15 minutes for the more dilute solutions. At first, it was necessary to orient ourselves; the denaturation was continued for 30 to 40 minutes, and aliquots of the solution analyzed at 10 minute intervals and returned to the container, and the experiment continued.

The measurement of the index of refraction was selected as an easy, quick, and accurate method for the determination of protein concentration. No claim is made for originality in using the index of refraction to determine protein concentration. This method has been used by many others (3), and we simply adapted it to our purposes. Considerable preliminary work was done to determine the accuracy and reliability of this method. It was found that over the range of temperatures used (23–26°) the indices of refraction of water and protein solution (up to 3.4 per cent) had the same temperature coefficient and, further, that the differences between the indices of refraction of water and those of the protein solutions were a straight line function of the protein concentration. This fact was established by drying to constant weight samples of varying dilutions of protein solution at 104° for 24 hours; the indices of refraction of these solutions had been previously determined. In a 35 cc. sample, which was the volume used in the denaturation experiments, 1 unit of the dipping refractometer corresponded to 0.0692 gm. of protein. It was possible to read the refractometer to 0.01 of a unit and to estimate to 0.001

of a unit. We could, therefore, estimate the concentration of the protein solution to an accuracy of  $6.92 \times 10^{-5}$  gm. of protein by this technique; this was sufficient for our purposes.

While 35 cc. of protein solution were selected as a convenient volume to use, through a wide range of volumes, the amount of denaturation is independent of the amount of solution in the container, as is shown by Table I.

The presence of cotton was necessary to remove the denatured protein from solution; otherwise part of the denatured protein was taken over by the drum and, accordingly, the new surface was not available for denaturation, and the amount of denaturation was less than it should be. It was found, however, that as long

TABLE I  
*Independence of Amount of Denaturation and Volume of Solution*

Concentration	Volume	R.P.M.	Protein denatured in 5 min.
<i>per cent</i>	<i>cc.</i>		<i>gm., <math>\times 10^3</math></i>
0.5	20	33.5	13.8
0.5	30	33.5	12.95
0.5	40	33.5	11.7
0.5	45	33.5	12.2
2.0	20	53	43.7
2.0	30	53	56.2
2.0	40	53	48.7
2.0	45	53	53.5

as a small amount of cotton was present, the denaturation reaction proceeded normally and the extent of denaturation was independent of the amount of cotton present, as is shown below.

35 cc. of 1 per cent solution at 53 R.P.M. for 5 minutes.

Weight of cotton <i>gm.</i>	Denatured protein <i>gm., <math>\times 10^3</math></i>
0.00	15.8
0.50	21.2
1.15	22.6
1.56	19.9

It was necessary for the interpretation of our calculations that all the denaturation observed took place at the solution-air surface and none at the drum-solution interface. This was demonstrated

to be the case by rotating a test-tube in a larger test-tube completely filled with protein solution. The larger test-tube was stoppered and the smaller test-tube rotated by means of a glass rod through the stopper in the larger tube. There was a strip of thick cloth between the smaller and larger tubes to scrape off any denatured protein if progressive denaturation occurred. Absolutely no denaturation was observed over 30 minute periods of rotation. We are safe, therefore, in assuming that all denaturation obtained upon rotating the porcelain drum exposed to air is occurring at the air-solution surface and that our calculations of thickness of the protein film refer to this surface only. No doubt there is adsorption and possibly denaturation on the drum surface, but this denaturation is not progressive and the denatured product cannot be removed by the cotton.

The rate of evaporation from the protein solution surface during rotation was greatly in excess of that observed for distilled water at the same speed of rotation and duration. There does not seem to be any simple relation between the amount of water evaporated and the amount of denaturation. As the surface film becomes thinner, owing to increasing speed of rotation or to decreasing protein concentration, the amount of evaporation is much larger for a given amount of denaturation. The quantity of water evaporated from a protein solution surface is much greater than that which could be accounted for on the basis of the difference in hydration of the surface-denatured and native protein. The weight of water evaporated from a protein surface in excess of that from a pure water surface varied from 22 gm. per gm. of denatured protein in the thicker film to 173 gm. in the thinner films. An experiment was conducted to determine whether there is a difference in rate of evaporation of a motionless protein solution and motionless pure water for the same surface, temperature, and relative humidity, but the rates were identical, indicating that the excessive evaporation from the denaturing protein is probably linked with the process of denaturation.

It seemed desirable to determine whether this large amount of evaporation would materially decrease the thickness of the layer of the solution on the wet drum as it rotated and accordingly increase the concentration of the protein on the drum, and in this way affect the experimental results. The amount of solution taken



along by the rotating drum was estimated by allowing a strip of blotting paper to run over the surface of the drum rotating at 26 R.P.M. and measuring the volume of water necessary to restore the apparatus to its original weight. Based on these results, the amount of water taken along by the drum for a 15 minute period was calculated and found to be 121 cc. The amount evaporated by a 0.1 per cent protein solution rotating at 26 R.P.M. and for 15 minutes is 4.32 cc., which would represent only a 3.5 per cent increase in the protein concentration in the layer of solution on the drum in excess of that in the bulk of the solution due to evaporation.

It is also important that the amount of denaturation is not limited by the amount of natural protein in the volume of solution on the drum. From the amount of water taken up by the blotting paper, the thickness of the layer of the solution on the drum rotating at 26 R.P.M. has been calculated to be about  $30\mu$ . 1 sq. cm. of the layer of a 1 per cent solution on the drum would, therefore, contain  $3.0 \times 10^{-5}$  gm. of protein. Now it was found by experiment that for a 0.916 per cent protein with the drum rotating at 26 R.P.M., the amount denatured per sq. cm. was  $5.9 \times 10^{-7}$  gm., or that only about 2 per cent of the protein was denatured. Therefore, this is not a limiting factor.

It has been assumed in the calculations reported in the discussion that the surface of the drum exposed per rotation is equal in area to that of the surface-denatured protein. If there were formation of bubbles or if the solution-air surface were thrown into waves by vibration or if there is dislodgement of the surface-denatured protein from the air-solution surface, these two areas would not be equal. A close examination of the protein solution surface as the drum rotated failed to disclose waves on the drum or formation of bubbles; everywhere on the roller the solution surface was completely smooth. Under these circumstances there seems little likelihood of surface-denatured protein being displaced from the surface. Surface-denatured protein is an insoluble, hydrophobic material and would not of its own accord leave an air-solution surface in favor of the soluble hydrophilic natural protein.

Since the protein used contained 7 per cent undenaturable substance, it was felt that these impurities might materially influence the state of affairs at the surface. To test this possibility, the following experiment was carried out. A certain volume of pro-

tein solution was heated on a water bath and the denatured protein filtered off. To the clear filtrate was added an equal volume of the original native protein solution. This solution then contained twice the amount of impurities as did the original solution. 35 cc. of this solution were placed in the apparatus and the drum rotated at 53 R.P.M. for 10 minutes and the amount of denaturation determined. A second solution was prepared by adding to the original, native protein solution an equal volume of pure water. This solution then contained the same concentration of native protein but only half the concentration of impurities as the first solution. 35 cc. of this solution were placed in the apparatus and the drum rotated at 53 R.P.M. for 10 minutes and the amount of denaturation determined. For the first solution containing a high concentration of impurities, 0.025 gm. of denatured protein resulted, and for the second, purer solution 0.023 gm. of denatured protein resulted; the two values are within experimental error of each other. This experiment indicates that the 7 per cent impurity does not materially affect the conditions at the surface.

It is known that protein denatures at a toluene-solution interface, and accordingly it was necessary to be certain that toluene was not present to form a toluene-solution interface during the experiment. The protein solutions were always pipetted from below the surface of the mother solution, and extreme care was exercised that no toluene was taken along with the aliquot. After the final dilutions were made, the surface of the solution was always examined by reflected light to be certain that it was not contaminated; the slightest trace of oil or solid impurities could be readily detected.

The denaturation experiments were conducted at room temperature, which varied between 23–26°. A measurement of the temperature coefficient of surface denaturation of egg albumin has been carefully made and will be reported on in the near future. Over this range of temperature, the temperature coefficient of surface denaturation is practically one and is not regarded as sufficient to affect the present results.

### *Results*

The amount, in gm., of protein denatured per minute is plotted in Fig. 1 against the number of revolutions of the drum in 1 minute for the several protein concentrations used. Two-thirds of the

experiments were checked by a subsequent determination. The data shown in Fig. 1 are the averages of the original and check determinations. The average deviation between the determinations and the average of these determinations was  $\pm 5.18$  per cent.

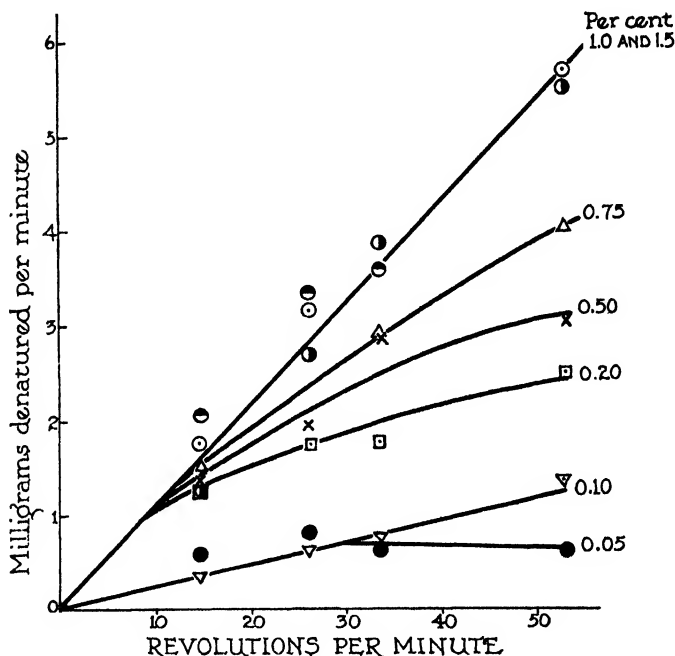


FIG. 1. The amount of denaturation per minute as a function of the speed of rotation. The concentration of egg albumin is represented as follows: ● 2.0 per cent, ○ 1.5 per cent, ⊙ 1.0 per cent, △ 0.75 per cent, × 0.50 per cent, □ 0.20 per cent, ▽ 0.10 per cent, ● 0.05 per cent.

The higher protein concentrations yielded a decreasing rate of denaturation with length of time of denaturation. All the results shown in Fig. 1 were obtained at the end of 5 minutes of denaturation, with the exception of those with the more dilute solutions, in which case the small amount of denaturation necessitated a longer interval. This short time reduced the error due to the excessive decreasing rate of denaturation to a minimum. Fig. 1

includes all data obtained on this protein solution with the exception of the rate at 26 R.P.M. and 0.75 per cent concentration. This point was rejected because both the original and repeated values were widely divergent and did not agree with each other, and the quantity of stock protein solution on hand did not permit rechecking. It was, therefore, deemed safer to reject all results for this point.

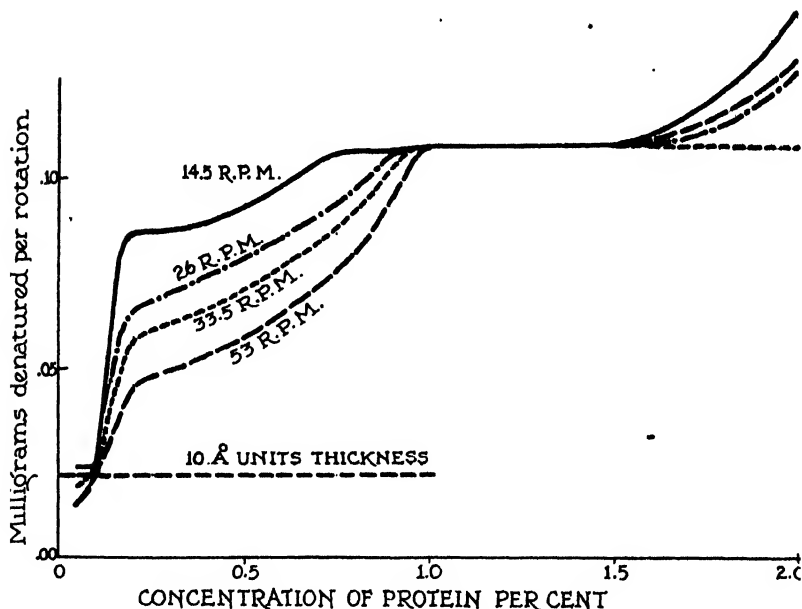


FIG. 2. The amount of denaturation per rotation as a function of the protein concentration for the several speeds of rotation used. The curves are drawn from points taken from the smooth curves in Fig. 1.

#### DISCUSSION

Figs. 2 and 3 show the amount of denatured protein produced per rotation of the drum plotted against speed of rotation and against concentration. These values, with the exception of the points indicated for 2.0 per cent, were calculated from the smooth curves of Fig. 1 and not directly from the experimental points.

Since we know the amount of protein denatured and its specific volume (4), it is possible to calculate the volume of the denatured

protein; also, since we know the area of the rotating drum and its rate of rotation, we can calculate the area exposed per minute. This is likewise the area occupied by the denatured protein. By dividing this area into the volume of denatured protein, we obtain the thickness of the film of denatured protein. From such calculations for the 0.1 per cent solution, we find as an average for the four speeds 10.6 Å. units. (The gm. of denatured protein which corre-

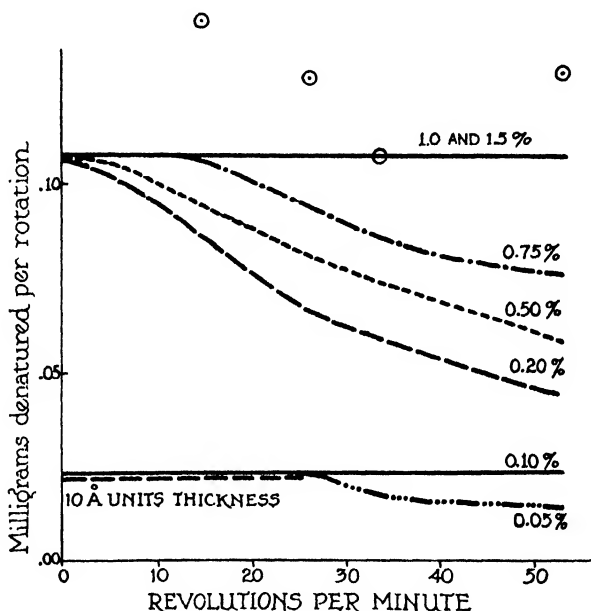


FIG. 3. The amount of denaturation per rotation as a function of the speed of rotation for the several concentrations used. The curves are drawn from points taken from the smooth curves in Fig. 1. The circles indicate the values for the 2.0 per cent solution.

spond to 10.0 Å. thickness have been indicated in Figs. 2 and 3.) This thickness is independent of the speeds of rotation and is probably the thickness of the film on a quiescent protein solution of this concentration. This is about the thickness of a peptide chain and its side groups, and is in good agreement with that found by Neurath (5) for a film of egg albumin spread on a buffer and measured by the Langmuir balance. The thickness of 10.6 Å.

indicates an unfolding of the peptide chain. This film is probably hydrophobic on its air side and hydrophilic on its water side, since the polar groups would be drawn into the water. This thickness at 0.1 per cent concentration is a very characteristic feature and was obtained in all the preliminary studies as well. Below 0.1 per cent protein concentration, the calculated thickness again becomes dependent on the speed of rotation as is shown clearly in Fig. 3; apparently, there is insufficient time to form a complete molecular film.

With concentrations greater than 0.1 per cent, the thickness of the film increases rapidly with an inflection at 0.2 per cent (Fig. 2). This feature also is highly characteristic and was obtained in all preliminary studies and led us at first to believe that this inflection indicated another layer of molecules which was about 25 Å. in thickness. We now believe it is safer to assume that the molecules absorbed beneath the first 10.6 Å. layer are still spherical and are accordingly 43 Å. in diameter but form an incomplete layer which accounts for an apparent thickness of less than 43 Å.

The inflection in the curves at 0.2 per cent (Fig. 2) is followed again by a gradually increasing rate of adsorption with increasing concentration until another inflection is encountered at 1 per cent concentration. This concentration along with the 1.5 per cent gives a limiting value for the rate of denaturation at a given speed. This limiting value must correspond to a favored configuration, since with 1 as well as 1.5 per cent concentration and for all speeds used, the amount of denaturation per rotation remained constant. In fact, curves of denaturation per rotation against speed of rotation (Fig. 3) can be extrapolated to zero speeds for 0.20, 0.50, 0.75, 1.00, and 1.50 per cent, and they all yield the same amount of denaturation per rotation.

A remarkable feature about this study is the speed with which the denaturation proceeds. For example, at 53 R.P.M. the film was exposed for only 0.785 second (account is taken of the fraction of the drum dipping into the solution), and yet for the 1.0 and 1.5 per cent solutions the reaction was complete. For the smaller concentrations down to 0.1 per cent this time is not sufficient, indicating that the rate of diffusion of the protein to the surface is important. The same situation is encountered below 0.1 per cent; here again the rate of diffusion is not sufficient to saturate

the surface. Actually, the time required for denaturation must be considerably less than 0.785 second, because the surface does not immediately become saturated with protein as the new surface is created, but the diffusion proceeds during the rotation and reaches saturation only after the drum has moved through part of its rotation. This short time is in marked contrast to the rather extended interval of 7 minutes reported for the formation of surface films on buffer solutions (5).

The author regards it as dangerous to attempt at this time a detailed discussion of the structure and thickness of the molecular layer or layers adsorbed beneath the 10.6 Å. layer. There are a few points, however, which are worth commenting on. The weight of protein removed from solution per rotation for 1.5 per cent is  $10.8 \times 10^{-5}$  gm. If we subtract the amount in the 10.6 Å. layer, we have  $8.4 \times 10^{-5}$  gm., which is 77.7 per cent of the total removed from solution. Now the cross-sectional area of spherical egg albumin molecules with a diameter of 43 Å. and a molecular weight of 40,500 and a weight of  $8.4 \times 10^{-5}$  gm. is 182.2 sq.cm.; close packing is assumed with no free spaces. 182.2 sq.cm. compare favorably with the actual area of the drum, which is 171.6 sq.cm., and lend color to the idea that the molecules in the second layer are spherical in shape and accordingly undenatured. It seemed worth testing this hypothesis with a direct experiment and to this end the following experiment was performed. 35 cc. of a 1.5 per cent solution of protein were placed in the apparatus and the drum rotated for 20 minutes at 26 R.P.M., and the amount of protein removed from solution was determined in the usual manner. Then the insoluble protein was removed from the apparatus and carefully washed a number of times with distilled water. After washing, it was dried at 105° for 24 hours and weighed. The experiment was repeated with 0.1 per cent initial protein concentration instead of 1.5 per cent concentration. 31.9 per cent of the protein removed from the 1.5 per cent solution was insoluble, while 93.5 per cent of that removed from the 0.1 per cent solution was insoluble. Evidently 68.1 per cent of the protein removed from a 1.5 per cent solution is soluble and undenatured. Thus a large fraction (88.3 per cent) of the protein of the second layer is undenatured, which is in accord with the suggestion that the molecules of the second layer are spherical in shape.

In connection with the above, Langmuir and Schaefer (6) have reported on the thickness of the film of egg albumin adsorbed from a 1 per cent solution of egg albumin on a surface of stearic acid, as determined by an optical method. They found the film to be 50 Å. in thickness. If we assume in our experiments that the protein on the surface is close packed, with no free spaces,  $6.3 \times 10^{-7}$  gm. of protein per sq. cm. corresponds to a total thickness of the surface film of 48.8 Å., which is in splendid agreement with the value found by Langmuir and Schaefer.

The determinations at 2 per cent were rather erratic, but in general showed a tendency to form a somewhat thicker surface layer than that pictured above.

No evidence is found in these experiments for a decreasing rate of denaturation with increasing concentration, as was found by Bull and Neurath (1). It seems probable that the decreasing rate as found by these workers was due to the higher viscosity of the more concentrated solutions, which resulted in less breakage of the surface when the more concentrated solutions were shaken.

Finally, it seems to the author that experiments of the type outlined in this paper should be of more interest to the biologist than studies of protein films on pure buffer solutions, since in living tissue we are dealing with solutions of protein and not with buffer solutions containing no protein. It is true that the present technique involves an extrapolation to zero speeds, but it still appears to be a more realistic approach to a study of protein films in biology.

#### SUMMARY

1. A new technique is described for the study of the kinetics of surface denaturation of proteins. A drum rotating at known speeds dips into a solution of protein and the resulting denaturation of protein is measured.

2. The amount of egg albumin denatured per unit area of surface has been determined as a function of rate of rotation of new surface as well as of the concentration of the solution. It has been found that both greatly affect the amount of denaturation.

3. The amount of denaturation per unit area of surface has been extrapolated to zero speed of rotation and yields an approximate picture of the conditions at the surface of a quiescent egg albumin



solution. At concentrations of 0.1 per cent and less, the surface film consists of a monomolecular layer of surface-denatured protein immediately on the surface with a thickness of about 10 Å. As the concentration is increased above 0.1 per cent, additional protein is adsorbed under the first 10 Å. layer. This second layer of protein contains as a limit 3.5 times the amount of protein in the first layer and is probably in an undenatured state.

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# THE EFFECT OF ADRENALECTOMY UPON BLOOD PHOSPHOLIPIDS AND TOTAL FATTY ACIDS IN THE CAT\*

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Various investigators working on the adrenal cortical hormone have centered their attention upon the abnormal exchange of water and salts across the tissue cell membrane which occurs after adrenalectomy. This phenomenon has led to the opinion that the cortical hormone is instrumental in maintaining normal cell permeability (1, 2). Alteration in the electrolyte content of erythrocytes following removal of the adrenals (2, 3) has shown that these cells reflect the absence of the cortical hormone. Further evidence of a change in blood cell permeability may be seen in the work of Reid (4) and of Roffo (5), who reported decreased fragility in the red blood cells of adrenalectomized animals. Furthermore, Blanchard (6) found that during adrenal insufficiency in cats, the proportion of cholesterol in the red blood cells to that in the plasma increased, and suggested that the normal permeability of the membrane was altered. The present paper extends the observations to phospholipids and total fatty acids, determined in cells and plasma before and after adrenalectomy in cats.

\* The material of this paper is taken from the dissertation presented by Eleanor H. Yeakel to the Faculty of the Graduate School of Bryn Mawr College in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The complete dissertation is on file in the Bryn Mawr College Library.

A part of this work was done at the Biological Laboratory, Cold Spring Harbor, New York, with facilities made possible by a grant from Bryn Mawr College.

*Procedure*

Thirteen cats were used in the experiments, all but four being males. Determinations of normal lipid values were made on blood samples taken from the normal cat in a postabsorptive condition. Bilateral adrenalectomy was performed in two stages under nembutal anesthesia (Abbott Laboratories). Following removal of the second gland, the animals were maintained for at least 5 days on cortical extract (Upjohn). During this time, and after injections were stopped, the blood was sampled as often as was considered safe for the animals; after severe insufficiency was evident, a terminal sample was obtained. The cats survived from 3 to 16 days, the average survival being 8 days.

Blood was obtained by cardiac puncture. Heparin was used in preference to other anticoagulants, since it does not change the relative cell-plasma volume and the relative amounts of lipids in serum and cells (7, 8). 6 cc. of blood were drawn, and 2 cc. of the whole blood were pipetted into a 3:1 mixture of alcohol and ether, and made up to 50 cc. for cold extraction. The remaining 4 cc. of blood were centrifuged at 2500 R.P.M. for 15 minutes, in graduated centrifuge tubes. The amount of cells and plasma was read as carefully as possible to obtain a hematocrit value, a procedure which gives fairly accurate results (9). The hematocrit reading served as a check upon the degree of hemoconcentration present, and also permitted calculation of the lipid in the red blood cells. 2 cc. of the plasma were pipetted into 3:1 alcohol-ether, and made up to 50 cc. The precipitate was filtered, and aliquots of the filtrate used for estimation of the total fatty acids and phospholipids, as in Boyd's modification of Bloor's oxidative method (10).

From the hematocrit readings and from the values for phospholipids and total fatty acids in the whole blood and plasma, similar values for the red blood cells were calculated.

*Results*

Data from the experiments on six of the animals are presented in Table I, and are similar to the results found for the remaining seven cats.

In all instances, the hematocrit value rose, following discon-

tinuance of hormone injections. This is in accord with similar data in the literature, showing that part of the fluid portion of the blood is lost during cortical insufficiency. It may be seen from Table I that the values for phospholipids and total fatty acids do not increase during the onset of symptoms. With the single exception of the final plasma phospholipid value for Cat 8, there is apparently a significant drop in lipids in the plasma following bilateral adrenalectomy. In Cat 5, in which the symptoms of adrenal insufficiency were slow to develop, the phospholipid and total fatty acid values remained high during the first 8 days following discontinuance of hormone injection. It may be seen also from Table I that the blood cells do not gain lipid. The only possible exception may be seen in the cell phospholipid values for Cats 8 and 9, in which increases of doubtful significance occur. The phospholipids and total fatty acids of whole blood and plasma showed, with the few exceptions noted above, a decrease from the normal value, in spite of the simultaneously occurring hemoconcentration.

Table I also shows the calculated values for the phospholipids and total fatty acids in the plasma and cells of 100 cc. of blood, together with the cell-plasma ratio for the lipid. -An analysis of these figures indicates a marked increase in cell-plasma lipid ratio during the development of the symptoms of adrenal cortical insufficiency. From an average of 1.5 for phospholipids and 1.4 for fatty acids, the ratio increases to approximately 3.2 for phospholipids (with extremes of 1.7 and 5.5) and to 2.4 for fatty acids (with limiting values of 1.5 and 3.1). Cat 5 may be taken as an example of such a rise in cell-plasma ratio; and as can be seen from Table I, this increase is a steady and progressive rise and not a terminal phenomenon.

#### DISCUSSION

It may be observed from the data recorded above that the plasma of adrenalectomized cats loses phospholipids and fatty acids. With the loss of water from the blood and the consequent concentration within it of certain of its elements, such as the proteins, it might be expected that the phospholipids, which are bound in part to the proteins and which do not pass through the kidney, would also become concentrated, and increase in amount

TABLE I  
*Phospholipids and Fatty Acids before and after Adrenalectomy*

Cat. No.	Date	Hematocrit		Phospholipid			Phospholipid in 100 cc. whole blood			Total fatty acids			Total fatty acids in 100 cc. whole blood			Remarks
		per cent	per cent	Whole blood	Plasma	Cells	mg.	Cell-plasma ratio	Whole blood	Plasma	Cells	mg.	Cell-plasma ratio	mg.	Cell-plasma ratio	
2	1938															
	Dec. 15	41	188.5	112.7	298	122.0	66.5	1.83	253.3	180.5	358	146.8	106.5	1.38	1st adrenal gland removed Dec. 6; 2nd gland removed Jan. 16. Recovery normal. Maintained with daily injections of cortical hormone until Jan. 30.	
	" 20	41	203.6	102.3	349	143.2	60.4	2.37	220.6	145.4	329	134.8	85.8	1.57	Staggered slightly in walking Feb. 6. Next day very weak. Killed by bleeding	
	1937															
	Jan. 20	40	160.6	127.4	211	84.2	76.4	1.10	241.9	210.6	269	115.5	126.4	0.91		
5	" 28	38	190.0	87.8	357	135.6	54.4	2.49	319.6	263.4	411	156.3	163.3	0.96		
	Feb. 2	43	204.0	86.4	360	154.8	49.2	3.15	324.5	265.8	402	173.0	151.5	1.14		
	" 5	51	216.8	83.7	345	175.8	41.0	4.29	300.4	217.8	380	193.7	106.7	1.82		
	" 7	56	162.2	45.3	254	142.3	19.9	7.15	229.9	166.9	280	156.5	73.4	2.13		
	" 5	37	204.0	154.4	288	106.7	97.3	1.10	333.4	289.6	408	151.0	182.4	0.83	1st gland removed Feb. 2; 2nd Feb. 15.	
	" 12	33	133.3	88.5	224	74.0	59.3	1.20	245.8	186.8	365	120.6	125.2	0.96	Recovery normal. Maintained on cortical hormone until Feb. 19. Appetite began to fail Feb. 28. Hind legs weak	
	" 18	40	158.2	73.8	285	113.9	44.3	2.57	261.1	204.9	346	138.2	122.9	1.12	Mar. 4. Mar. 7, unable to stand; body temperature low. Died 5 hrs. after last bleeding	
	" 24	47	181.2	110.0	261	122.9	58.3	2.11	283.9	219.0	357	167.8	116.1	1.45		
	" 27	58	225.1	123.1	299	173.4	51.7	3.35	307.7	255.9	345	200.2	107.5	1.86		
	Mar. 3	60	195.7	75.7	276	165.4	30.3	5.46	315.0	196.1	394	236.6	78.4	3.02		
7	" 7	60	155.3	64.7	216	129.4	25.9	5.00	242.9	172.1	290	174.1	68.8	2.53		
	Jan. 19	35	166.0	97.3	294	102.8	63.2	1.63	227.4	146.2	378	132.4	95.0	1.39	1st gland removed Mar. 22; 2nd Mar. 29.	
	Mar. 25	32	131.0	90.6	217	69.4	61.6	1.13	216.5	131.4	397	127.1	89.4	1.43	Recovery normal. Maintained on cortical hormone until Apr. 4. Refused food Apr. 8. Hind legs weak Apr. 9.	
	Apr. 1	35	156.8	82.8	294	103.0	53.8	1.91	225.0	95.1	466	163.2	61.8	2.64	Unable to stand Apr. 10. Killed by bleeding	
	" 6	42	106.3	77.7	146	61.2	45.1	1.36	214.5	120.0	345	144.9	69.6	2.08		
	" 9	43	89.3	40.2	154	66.4	22.9	2.90	212.6	96.7	366	157.5	55.1	2.86		
	" 10	39?	93.9	45.0	170	66.4	27.5	2.41	185.9	95.2	328	127.8	58.1	2.20		

8	Mar. 19	34	111.3	61.2	209	70.9	40.4	1.75	251.6	161.9	426	144.7	106.9	1.35	1st gland removed Mar. 22; 2nd Mar. 29. Recovery normal. Maintained on cortical hormone until Apr. 4. Appetite poor Apr. 8. Weak in hind legs Apr. 12. Found dead Apr. 14, 14 hrs. after last bleeding
	" 25	34	102.7	59.0	227	63.8	38.9	1.64	203.8	144.1	320	108.7	95.1	1.14	
	Apr. 1	38	124.0	80.6	195	74.0	50.0	1.48	225.8	114.0	482	155.1	70.7	2.19	
	" 6	38	144.8	65.0	275	104.5	40.3	2.59	245.2	114.0	459	174.5	70.7	2.47	
	" 9	42	135.1	47.3	256	107.7	27.4	3.92	236.3	137.1	373	156.8	79.5	1.97	
9	" 13	47	154.7	68.3	245	119.9	34.8	3.45	233.4	137.8	333	163.1	70.3	2.32	1st gland removed Mar. 22; 2nd Mar. 29. Recovery normal. Maintained on cortical hormone until Apr. 3. Gave no indication of adrenal insufficiency except slight sluggishness of movements Apr. 10 in the morning. Found dead that afternoon
	Mar. 19	32	108.8	73.6	181	57.8	51.0	1.13	284.7	201.3	462	147.8	136.9	1.08	
	" 25	28	120.1	80.9	221	61.8	58.3	1.06	271.0	178.1	510	142.8	128.2	1.11	
	Apr. 1	24	147.7	98.8	303	72.6	75.1	0.97	245.6	205.0	374	89.8	155.8	0.57	
	" 6	23?	150.2	70.0	419	96.3	53.9	1.79	233.4	167.7	286	65.7	129.1	0.51	
10	" 9	30	116.2	61.3	265	79.4	36.8	2.16	195.4	141.5	368	110.5	84.9	1.30	1st gland removed Mar. 22; 2nd Mar. 29. Recovery normal. Maintained on cortical extract until Apr. 4. Appetite poor, movements listless Apr. 6. Body temperature low Apr. 7. Killed by bleeding
	Jan. 19	36	168.1	85.0	316	113.7	54.4	2.09	253.8	132.0	470	169.3	84.5	2.00	
	Mar. 25	36	133.2	79.7	228	82.2	51.0	1.61	252.7	200.7	345	124.2	128.5	0.97	
	Apr. 1	42	132.9	84.4	200	83.9	49.0	1.71	232.2	109.5	306	168.7	63.5	2.66	
	" 6	44	93.1	29.7	174	76.5	16.6	4.61	275.6	121.8	471	207.4	68.2	3.04	
	" 7	53	85.8	30.0	116	61.7	14.1	4.38	201.0	102.9	288	152.6	48.4	3.15	

over the normal values for a given volume of blood. Baumann and Holly (11) reported that the lipid phosphorus rose steadily in the whole blood of rabbits suffering from sublethal cortical deficiency. Harrop and Weinstein (12), however, found no consistent changes in the phospholipids of the serum of adrenalectomized dogs; in severe insufficiency the levels were sometimes low and sometimes normal. The data of the present paper likewise fail to show the expected increase in phospholipids and total fatty acids. The severe bleeding to which the cats were subjected might be expected to produce, if anything, a rise in blood lipids (13). On the contrary, it seems apparent that some of the lipids are removed from the blood.

Comparison of the distribution of lipids between cells and plasma shows that the ratio of cell phospholipid to plasma phospholipid, and also of cell total fatty acids to plasma fatty acids, increases over the normal value. Following reduction of plasma phospholipid, the presence within the red blood cells of an amount of lipid well in excess of that in the surrounding plasma may seriously affect the membrane permeability, and account for the abnormal salt exchanges (2, 3), decreased erythrocyte fragility (4, 5), and decreased ratio of plasma sugar to cell sugar (14) seen after adrenalectomy. Blanchard (6) demonstrated a similar abnormal distribution of cholesterol between cells and plasma following adrenalectomy in cats, and suggested the possibility that this abnormal cholesterol might alter membrane permeability.

Calculation of the loss of the phospholipids and total fatty acids compared to that of water shows that the fluid and fats do not disappear proportionally. Consequently, the phenomenon involves more than a mere passage across the membrane of a watery solution of phospholipids at the same concentration at which they occur in the plasma.

The fate of the disappearing phospholipids is not clear. Their hydrolysis seems unlikely, since there is no evidence that the products of hydrolysis—fatty acids, free or combined—accumulate in the blood. Their storage is not probable in the light of evidence presented by Verzar and Laszt (15) that after adrenalectomy in rats, fat transportation to, and mobilization from, depots is inhibited. Moreover, the large number of recent papers which show that ketosis from various causes is inhibited partly or wholly

after adrenalectomy (16) suggest that the adrenalectomized animal would be incapable of catabolizing the fatty acids in question.

Another possibility is that the phospholipids leaving the blood may accumulate in the tissues. A similar shift of lipids is reported in the paper of Thaddea and Fasshauer (17), who showed an increased blood cholesterol to occur after bilateral adrenalectomy in cats, which fell to normal when cortical extract was injected. The cholesterol content of liver and voluntary muscle correspondingly fell and rose in these respective circumstances. These changes in lipid content of the tissues appear at first glance to be directly opposed to the results of Verzar and Laszt; but the latter investigators dealt with the ordinary storage and transfer of fats which they believed to include phosphorylation, while the process of loss of phospholipids from the blood in the absence of the cortical hormone may involve an entirely different mechanism.

Sinclair (18) pointed out that certain investigators consider a proper balance between phospholipids and cholesterol to be important in the water-salt balance of the body, an excess of phospholipid exerting a hydrophilic effect. Dahmlos and Solé (19), for example, demonstrated that intravenous injections of phospholipid emulsions in rabbits lead to hydremia. It is possible that the phospholipids, following adrenalectomy, are able to penetrate to the inside of cells of tissues such as endothelium, muscle, etc., or in some manner accumulate at the cell boundaries. If this phenomenon be accompanied by a loss of tissue cholesterol, as claimed by Thaddea and Fasshauer, then the ratio between these lipids would be altered in the direction necessary for the passage of water from the blood into the tissues, such as actually occurs after adrenalectomy.

#### SUMMARY

1. Determinations of phospholipids and total fatty acids were made upon the whole blood and plasma of cats before and after adrenalectomy. From these values, the phospholipid and total fatty acid content of the red blood cells was calculated.

2. In spite of hemoconcentration revealed by hematocrit readings, the lipids in the whole blood, plasma, and cells decreased. It was concluded that lipid was lost from the plasma.

3. The distribution of phospholipids and total fatty acids



between cells and plasma in 100 cc. of whole blood was calculated. During insufficiency, the ratio of lipids between cells and plasma increased markedly.

4. Theories to account for the loss of lipid from the plasma, and the possible effects of this lipid change upon water balance, were discussed.

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## THE RELATION OF ALANINE TO GROWTH\*

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Despite the fact that alanine has been recognized as a component of proteins for almost 60 years, no reliable information is to be found in the literature regarding its nutritive rôle. This is to be accounted for by the fact that hitherto no method was available for the preparation of diets devoid of this amino acid, but adequate in other respects. By the use of mixtures of highly purified amino acids in place of proteins, such a ration may be formulated with ease. The present paper describes the growth behavior of animals upon a régime of this nature.

### EXPERIMENTAL

The composition of the amino acid mixture (Mixture XII-b) is shown in Table I. As will be observed, it was devoid of threonine and alanine. These were incorporated in the diets separately. All of the amino acids were analytically pure. In order to insure the complete absence of alanine, each of the ten *natural* acids was recrystallized three times after it yielded correct analytical values.

The make-up of the diets is shown in Table II. Diet 1 contained 2 per cent of *dl*-alanine, while Diet 2 contained none. Both supplied 18 per cent of "effective" amino acids including glucosamine. The vitamin B factors were furnished to each animal in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily intake of nitro-

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gen from these sources amounted to approximately 4 mg., and was the only nitrogen of unknown kind in the rations.

Two litters of young rats were employed in the growth studies. The feeding trials with Litter 1 were terminated after 20 days,

TABLE I  
*Composition of Amino Acid Mixture*

	Mixture XII-b	
	Active amino acids	As used
	gm.	gm.
Glycine.....	3.00	3.00
Alanine.....	0	0
Valine.....	8.00	16.00*
Leucine.....	9.00	18.00*
Isoleucine.....	4.00	8.00*
Norleucine.....	1.25	2.50*
Proline.....	8.00	8.00
Hydroxyproline.....	2.00	2.00
Phenylalanine.....	3.90	7.80*
Glutamic acid.....	22.00	22.00
Aspartic ".....	4.10	4.10
Serine.....	1.50	3.00*
Tyrosine.....	6.50	6.50
Cystine.....	1.25	1.25
Histidine.....	2.52	
" monohydrochloride.....		3.40
Arginine.....	5.25	
" monohydrochloride.....		6.35
Lysine.....	7.70	
" monohydrochloride.....		19.25*
Tryptophane.....	2.25	2.25
Methionine.....	1.75	3.50*
Sodium bicarbonate.....		12.86
	93.97	149.76†

\* Racemic acids.

† 1.594 gm. of mixture are equivalent to 1.0 gm. of "effective" amino acids.

and with Litter 2 after 28 days. As has been pointed out frequently in the past, many of our experiments involving the use of mixtures of purified amino acids have been of relatively short

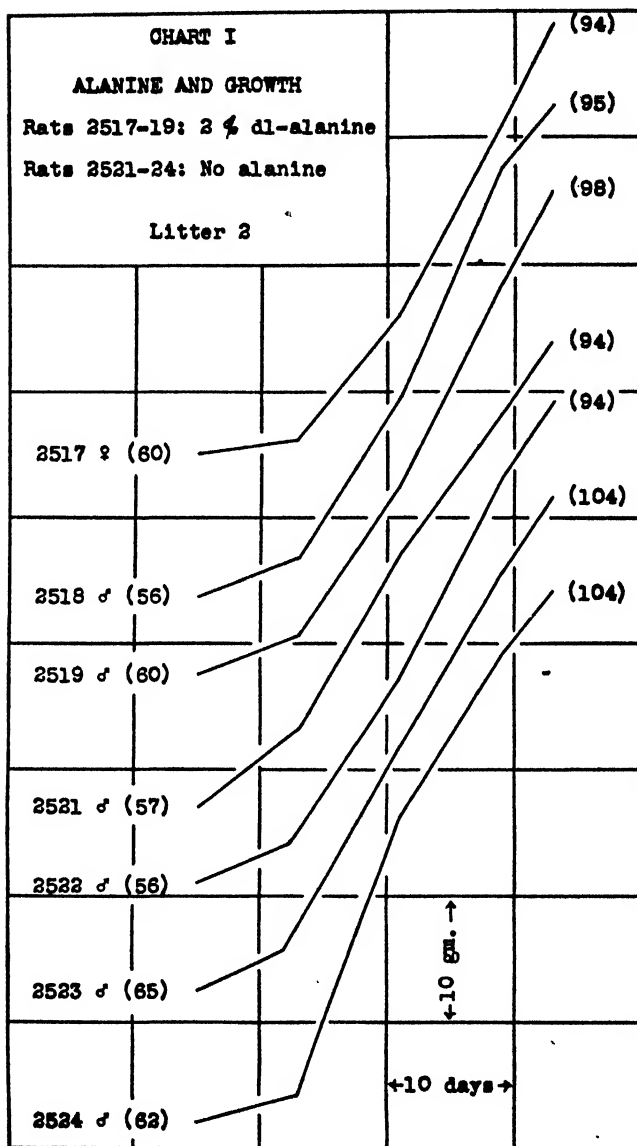


CHART I. The numbers in parentheses denote the initial and final weights of the rats.

TABLE II  
*Composition of Diets\**

	Diet 1	Diet 2
	<i>gm.</i>	<i>gm.</i>
Amino acid Mixture XII-b . . . . .	24.7	26.3
Threonine . . . . .	0.7	0.7
Glucosamine hydrochloride ( <i>d</i> -) . . . . .	1.0	1.0
Sodium bicarbonate . . . . .	0.4	0.4
Sucrose . . . . .	15.0	15.0
Dextrin . . . . .	19.2	19.6
Lard . . . . .	26.0	26.0
Salt mixture† . . . . .	4.0	4.0
Agar . . . . .	2.0	2.0
Cod liver oil . . . . .	5.0	5.0
Alanine ( <i>dl</i> -) . . . . .	2.0	0
	100.0	100.0

\* Each diet contained 18 per cent of "effective" amino acids, including glucosamine. The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily intake of nitrogen from these sources amounted to approximately 4 mg.

† Osborne and Mendel (1919).

TABLE III  
*Total Changes in Body Weight and Total Food Intakes of Experimental Animals*

Litter No.	Rat No. and sex	Duration of experiment	Total increase in weight	Total food intake	Supplement
		<i>days</i>	<i>gm.</i>	<i>gm.</i>	
1	2473 ♀	20	25	94	Alanine
	2474 ♀	20	26	100	"
	2475 ♂	20	28	106	"
	2476 ♂	20	28	93	"
	2477 ♀	20	26	102	No alanine
	2478 ♀	20	28	97	" "
	2479 ♀	20	27	90	" "
	2480 ♂	20	25	101	" "
	2481 ♂	20	37	122	" "
	2517 ♀	28	34	133	Alanine
2	2518 ♂	28	39	149	"
	2519 ♂	28	38	140	"
	2521 ♂	28	37	161	No alanine
	2522 ♂	28	38	155	" "
	2523 ♂	28	39	183	" "
	2524 ♂	28	42	163	" "

duration. This has been necessary because of the tremendous cost of the materials. At a later date prolonged experiments with simplified mixtures will be undertaken.

Inasmuch as the results were quite uniform, the growth curves of only one litter (Litter 2) are reproduced in Chart I. In Table III are recorded the total changes in body weight and the total food intakes of the animals of both litters. During the first few days of the tests the rats grew quite slowly. Later, as will be seen from Chart I, the rates of gain increased in both groups. A temporary inhibition in growth occurs not infrequently following the transfer of animals from a stock ration to one containing mixtures of amino acids. Indeed, this is observed occasionally when the experimental diets carry 18 per cent of whole casein in addition to all non-protein dietary essentials. Why this should take place in certain individuals and not in others is not clear. In the present experiments the initial lags in growth were not associated with the alanine content of the food. The behavior of the animals which received this amino acid is comparable in every respect to that of their litter mates which were deprived of it. *The data demonstrate that the omission of alanine from the food is without influence upon growth.* Evidently this amino acid, like glycine and serine (McCoy and Rose, 1937), but in contrast to leucine and isoleucine (Womack and Rose, 1936), is not an indispensable component of the food.

#### SUMMARY

By the use of diets devoid of proteins, but containing mixtures of highly purified amino acids, *alanine has been shown to be a dispensable component of the food.* Rats deprived of this amino acid increase in body weight just as rapidly as do controls receiving a similar ration supplemented with alanine.

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## ELECTROPHORESIS OF POSTERIOR PITUITARY GLAND PREPARATIONS

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(Received for publication, November 23, 1937)

Attempts to isolate the physiologically active principle or principles of the posterior lobe of the pituitary gland in the pure form have involved, for the most part, successive fractionations with various precipitants and solvents. By such procedures highly potent, non-crystalline preparations have been obtained containing, on the one hand, high pressor activity with relatively little oxytocic activity and, on the other hand, high oxytocic activity with relatively little pressor activity (1, 2). However, the continued application of these methods for further purification of the principles has not yielded material of greater potency nor given rise to crystalline preparations. An entirely new approach to the problem seemed, therefore, to be warranted. In the event that a new technique should, in itself, prove inadequate for the isolation of the pure active material, it was thought that the application of the existing fractionation methods to the product obtained thereby might eventually lead to isolation of the principles. In other words, it would be logical to assume that a radically different purification procedure would produce active preparations differing qualitatively with respect to the impurities present from those obtained heretofore.

The work of Williams and his coworkers (3-6) on "fractional electrical transport," with a multiple celled electrophoretic apparatus, strongly suggested the possibility that this method of approach might be useful in effecting a separation of the active pituitary substances from their accompanying impurities and even

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in effecting a separation of the principles from one another on a scale large enough for actual preparative purposes. Since previous work has indicated that these principles, although complex, have molecules smaller than proteins, this approach appeared all the more attractive (7).

In the apparatus described by Williams, direct current at high voltage was passed through a series of beakers connected by means of siphons. The material to be electrolyzed was placed in one or more of the beakers and the remaining cells were filled with distilled water. In such an electrophoretic set-up, according to Williams (3), "A gradient of pH values is established between the anode cell and the cathode cell and an ampholyte tends to move toward the cell possessing the pH value which approximates the isoelectric point of the ampholyte." It is clear that advantage may also be taken of the relative rates of migration of the substances involved.

It is the purpose of this paper to present the procedure which we have developed for the application of electrophoresis to the pituitary problem and to describe the apparatus which we have used for this work. The electrophoretic behavior of the pressor and oxytocic activities in a long train set-up will also be described, since the results obtained are very encouraging with respect to the utilization of the technique in the purification of these principles on a preparative scale. Further work along these lines is now in progress.

#### EXPERIMENTAL

In the electrophoresis of his materials, Williams found that relatively high voltages and dilute solutions were necessary for obtaining satisfactory fractionation in reasonable periods of time. In our application of Williams' procedure to pituitary preparations, high voltages were also found necessary. Preliminary attempts to carry out the electrophoresis at 220 volts resulted in only negligible movement of activity out of the starting cell in periods as long as 80 hours. With this condition established, an exploratory high voltage experiment was carried out with the facilities kindly placed at our disposal by Dr. F. E. Allison of the Bureau of Chemistry and Soils, United States Department of Agriculture. The crude active powders which were utilized were prepared from

posterior pituitary glands by extractions and fractionation procedures similar to those described by Kamm *et al.* (1). 10 mg. of active material in the form of a powder containing 8 pressor and 8 oxytocic units per mg. were dissolved in 20 cc. of distilled water and placed in the center cell of a five cell set-up similar to the seven cell train shown in Fig. 1. Each of the remaining cells contained 20 cc. of distilled water. The siphons connecting the cells were filled by gentle suction on the manifold shown in Fig. 1, and the carbon electrodes were connected to the high potential source. After 70 hours of electrolysis at 1000 volts, the cell contents were removed and assays were made for both pressor and oxytocic activity. Pressor assays were carried out on cats under deep amytal anesthesia, and oxytocic assays were based on the

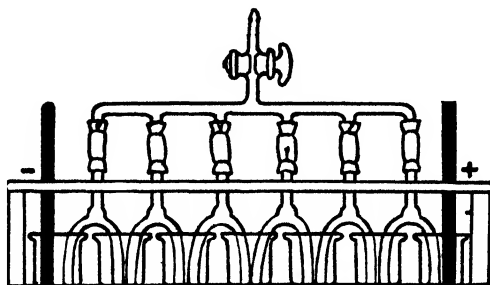


FIG. 1. Small capacity electrophoretic set-up. Seven 50 cc. beakers

depressor effect of the oxytocic principle on the blood pressure of the hen under urethane, as used in previous studies reported from this laboratory (8). The distribution of the activities in this experiment is shown in Table I.

As can be seen from the data, the pressor activity left the center cell completely and accumulated in the cathode cell. Oxytocic activity, however, was found in both the cathode and the center cells, indicating that its movement during the electrophoresis was less complete and suggesting the probability of a slower rate of migration for the oxytocic principle. Within the error of the assay, both activities were quantitatively recovered, showing that no inactivation or destruction had taken place. A definite pH gradient was established between the cathode and

anode cells. The ratio of activity to solids present in Cells 1 and 3 indicated that considerable fractionation of the starting material had been accomplished.

This experiment demonstrated that the two principles migrated toward the cathode, as was to be expected from data in the literature (9, 10). Furthermore, the increase in potency of the material collected from the cathode cell strengthened the belief that the procedure would serve as an effective purification method. Other experiments in which samples were removed from the cathode cell at intervals during the electrophoresis gave additional indications that the two principles differed in their speeds of migration, the pressor traveling more rapidly than the oxytocic.

TABLE I  
*Distribution of Activities in Preliminary Experiment*

Cell No.	Before electrophoresis			After electrophoresis			
	Pressor units	Oxytocic units	Solids	Pressor units	Oxytocic units	Solids	pH
			<i>mg.</i>			<i>mg.</i>	
1 (Cathode)	0	0	0	100	60	2.8	8.2
2	0	0	0	0	0		6.1
3	80	80	10	0	20	5.0	4.7
4	0	0	0	0	0		3.3
5 (Anode)	0	0	0		0		2.6

The construction of an apparatus to apply the procedure to large scale work seemed, therefore, to be warranted. It was also felt that in order to take full advantage of the different migration rates of the two principles, a longer path for migration was desirable. The accomplishment of these two objectives meant increasing the capacity of the electrophoretic train and devising a source of higher potential.

The electrophoretic set-up of increased capacity with 400 cc. beakers, which we have used in the larger scale work, is shown in Fig. 2.

For the source of current a high potential unit capable of supplying a variable, direct current potential up to 15,000 volts was designed and constructed in cooperation with the electrical engi-

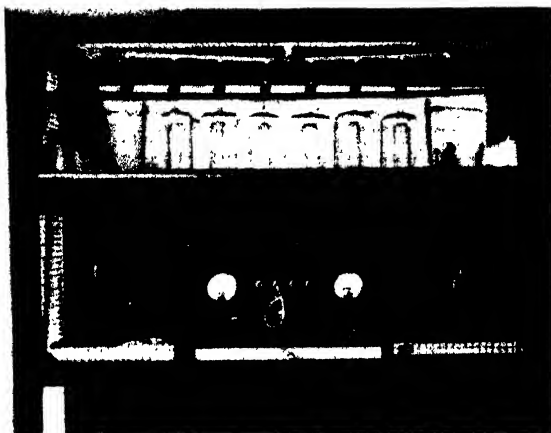


FIG. 2. Large capacity electrophoretic apparatus. The siphons are of 25 mm. Pyrex tubing and extend to within 2 to 3 mm. of the bottoms of the beakers. Each set of siphons has been provided with a connecting manifold, so that all of the siphons may be filled and emptied simultaneously. Suitably perforated caps of rubber sheeting may be used to cover the cells, thereby minimizing evaporation and eliminating dust contamination. The electrodes consist of cylindrical sticks of pure carbon, 6 mm. in diameter. The cabinet has been divided into two compartments by means of a shelf. The lower half houses the electrical apparatus and the instrument panel, while the upper half serves as a platform for placing and connecting the electrophoretic set-ups. Except for the glass window covering the upper half of the front, the cabinet, including the hinged top, is enclosed by means of a wire grill which is kept grounded. A safety device, consisting of a switch under the hinged top, which automatically opens the high voltage transformer primary when the top is lifted, is included to prevent accidental injury to the operator. As the apparatus is arranged, the high voltage outlets are not accessible unless the top of the cabinet is lifted, whereupon the circuit is broken.

neering department of the University.<sup>1</sup> The circuit employed for developing the required voltage from a 110 volt alternating current source is given in Fig. 3. Full wave rectification of the alter-

<sup>1</sup> We are indebted to Professor Norman Bruce Ames and Professor Alfred Ennis, Department of Electrical Engineering, George Washington University, for constructing the electrical apparatus and for aiding with valuable advice on numerous occasions.

nating current was accomplished through the use of two General Electric FP-85 kenotron tubes, which, with the use of suitable condensers, wired as shown in Fig. 3, afforded a voltage-doubling system. Variation of the output voltage was effected by means

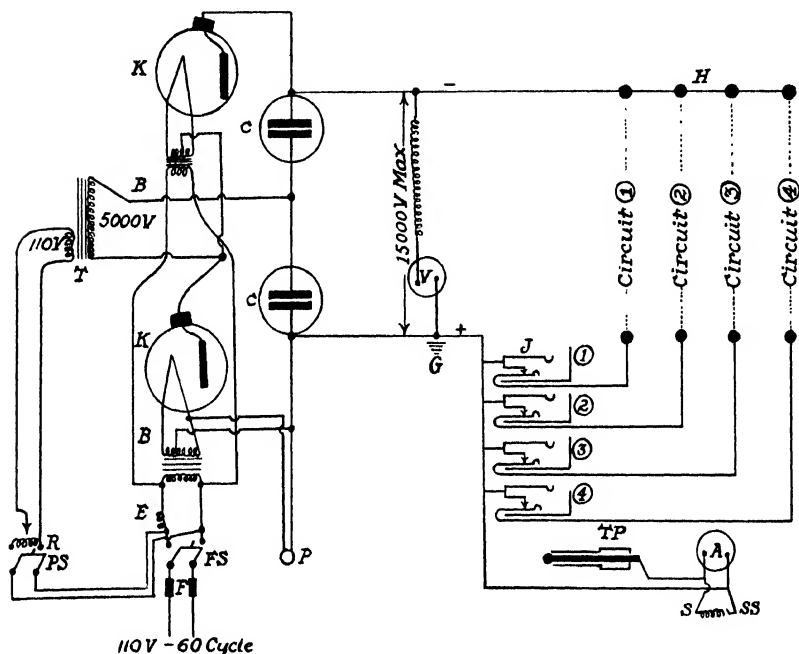


FIG. 3. Diagram of electrical circuit. *A*, Weston model No. 600 microammeter; *B*, 10 volt filament transformers; *C*, General Electric pyranol 7500 volt, 1.0 microfarad capacitors; *E*, current-limiting resistor; *F*, fuses; *G*, ground to cabinet grill; *J*, jacks; *K*, General Electric FP-85 kenotron tubes; *P*, pilot lamp; *R*, variac voltage adjustment; *S*, 16.7 ohm ammeter shunt for milli-ampere range; *SS*, shunt-opening switch; *T*, 5000 volt, 0.025 ampere plate transformer; *V*, Weston model No. 301 voltmeter; *PS*, plate switch; *FS*, filament switch; *TP*, telephone plug; *H*, porcelain insulators.

of the variac resistance shown, which permits accurate adjustment of the voltage between zero and maximum practically independent of the loading. Four separate, high voltage circuits were provided so that four electrophoretic set-ups could be run simultaneously. The current through each of these circuits was measured by

means of the system of jacks and the ammeter plug shown in Fig. 3. The circuits are normally closed through the jacks, but the insertion of the ammeter plug connects the ammeter without opening the circuit.

As already stated, the preliminary experiments had given encouraging results from the standpoint of purification and had indicated that the two principles possessed some degree of difference in their rates of migration. It was decided, therefore, to carry out an experiment with an exceptionally long series of beakers, so that the relative migration rates of the principles could be more clearly brought out and thus a better understanding

TABLE II

*Units of Activity in Starting Cell, Cathode Cell, and Total Cells in Nineteen Cell Experiment*

Day.....	1	2	3	4	5	6	8	10	13	17
Pressor										
Cathode cell (No. 1).....	0	0	133	350	665	1470	1610	1785	1295	945
Starting " ( " 17).....	3990	3200	3020	2532	2160	1750	525	56	48	28
Total (19 cells).....	4847	4519	5103	4606	4400	5024	3530	2552	2391	1580
Oxytocic										
Cathode cell (No. 1).....	0	0	0	53	123	238	385	735	875	805
Starting " ( " 17).....	2380	2490	2010	1663	1660	1260	420	35	11	
Total (19 cells).....	2512	3217	3030	3054	2872	2860	2143	1411	1185	847

of the general behavior of the two principles might be obtained. Furthermore, in such a long train the effect of diffusion on the observed movement of the activities would be minimized. In this way it was hoped that the question of differential migration could be definitely settled.

Nineteen 400 cc. beakers were connected in series by means of eighteen siphons. 300 mg. of a preparation<sup>2</sup> containing 5610 units of pressor and 3090 units of oxytocic material dissolved in 350 cc. of distilled water were placed in Cell 17, the pH of the solution

<sup>2</sup> We wish to express our appreciation to Dr. Oliver Kamm of Parke, Davis and Company, for so generously supplying us with the frozen glands from which we prepared our material.

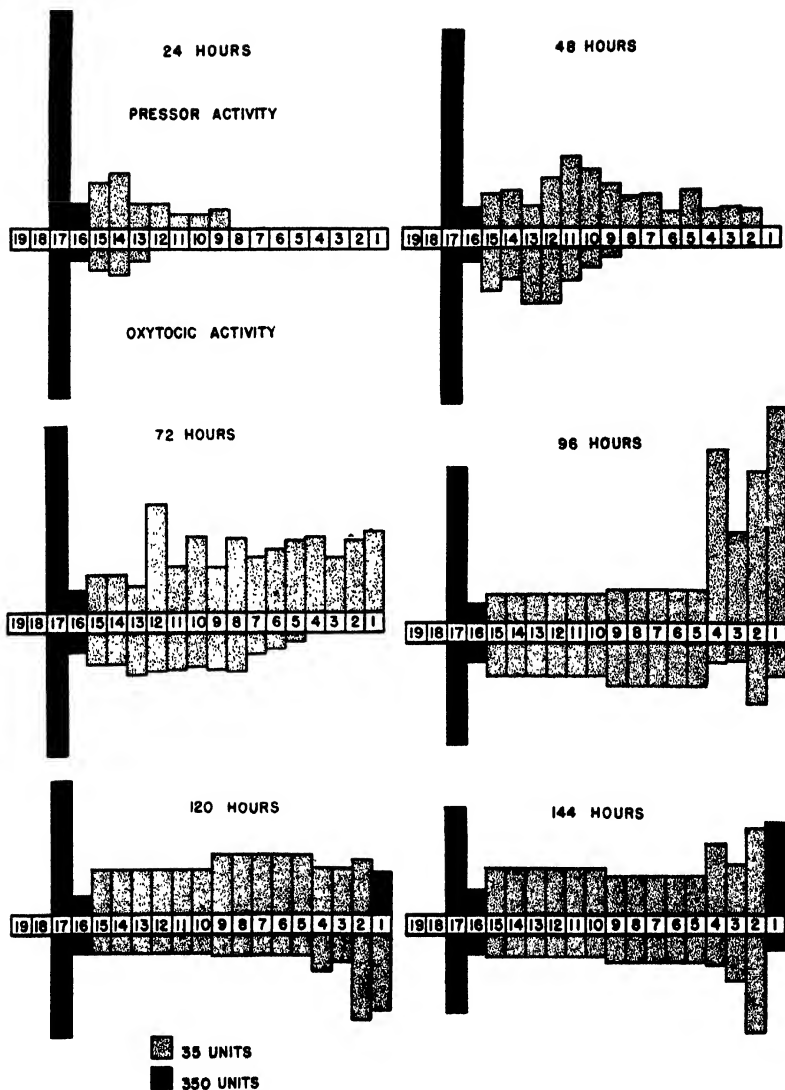


FIG. 4. Nineteen cell experiment. The pressor and oxytocic activity present in each cell at the end of each day of electrophoresis is shown graphically. The numbered squares represent the nineteen cells of the electrophoretic train. The active material was placed in Cell 17 at the start of the experiment. Cell 1 is the cathode cell. The values shown for Cells 5 to 9 and for Cells 10 to 15 for the 4th, 5th, and 6th days were obtained by assaying combined aliquots from each of these cells and dividing the total by the number of cells included in the combination.

being 4.0. (The cells have been designated numerically, the cathode cell being Cell 1.) The remaining cells were filled with 350 cc. of distilled water, and the siphons were filled by suction on the manifold. Electrophoresis at 14,000 volts was continued for 17 days, the contents of each cell being assayed daily for the first few days and at frequent intervals thereafter, for both pressor and oxytocic activity. The results are given in Fig. 4 and in Table II.

The results of the nineteen cell experiment strikingly demonstrate a differential migration of the pressor and oxytocic principles in the preparation used. At the end of 24 hours of electrophoresis, pressor activity was found to have moved as far as Cell 9, while the oxytocic activity had only progressed as far as Cell 13. At the end of the 2nd day, this difference was even more marked, pressor activity having reached Cell 2, while the slower moving oxytocic activity had migrated only as far as Cell 9. After 72 hours, pressor activity had already begun to accumulate in the cathode cell, but no oxytocic activity could be detected in any cell nearer the cathode than Cell 5. Assays made after the 4th day showed that at this time oxytocic activity had finally reached Cell 1, and that the pressor activity present was practically 3 times that of the previous day. After the 5th and 6th days both activities were accumulating rapidly in Cell 1 at relatively constant rates. After 96 hours, when oxytocic activity was first found in Cell 1, the pressor to oxytocic ratio in that cell was 7:1; after 120 hours this ratio was 5:1; and at 144 hours, 6:1, indicating that the pressor principle was accumulating in the cathode cell at an average rate 6 times that of the oxytocic principle.

During the first 6 days of electrophoresis a regular decrease of both the pressor and oxytocic activities in the starting cell paralleled the movement and accumulation of the principles in the cathode cell. The cells between the starting and cathode cells contained small, but relatively constant, amounts of activity. Totals of the assay values for all cells on each of the 6 days showed that recovery of the starting activity was complete for both principles, so that preferential destruction of the oxytocic activity could not account for its slower accumulation in the cathode cell. This fact is seen on examining Table II where the pressor and oxytocic assay values for the cathode cell, the starting cell, and



the totals for all cells are given for the entire 17 days of electrophoresis.

Further examination of Table II, however, reveals that after 6 days the total pressor and oxytocic activity in all cells suffered a decided decrease which continued for the remainder of the experiment. Analysis of the data from this and similar experiments indicates that this destruction of activity takes place in both the cathode cell and in the starting cell, in the cathode cell perhaps as the result of the alkalinity (pH 9.0) or as the result of some electrode reaction, and in the starting cell as a result of the growth of molds. No preservative could be added without danger of affecting the animal assays. While this obvious destruction or inactivation makes it impossible to draw any further conclusions regarding the relative rates of migration of the two principles after the 6th day of electrophoresis, the results of the first 6 days are nevertheless truly representative of this behavior, since in that period no inactivation or destruction of either activity took place. Even though destruction was taking place after the 6th day, electrophoresis was continued for the sake of gaining information concerning the rate of this destruction and of determining where the destruction was taking place.

For routine work, the fact that activity is destroyed after the 6th day under the above conditions is of little importance, since in routine work prolonged periods of electrophoresis are unnecessary. We have found that a seven cell series of 400 cc. beakers and an electrophoretic period of 60 to 70 hours are very effective for obtaining purification of the principles. Furthermore, steps can be taken to prevent undue destruction of activity in the cathode cell. The inactivating effect of long exposure to alkali in the cathode cell can be avoided by replacing the solution at intervals with distilled water. Likewise, electrode reactions can be eliminated by isolating the electrode at the cathode end in a separate compartment, as suggested by Williams (6) and Das, Ghosh, and Guha (9).

Numerous other experiments in which electrophoretic trains of various lengths and in which varying concentrations of active material have been used amply confirm the results and conclusions obtained in the nineteen cell experiment which has been described.

# SUMMARY

The apparatus and the procedure which have been used in studying the electrophoresis of posterior pituitary preparations have been described.

The results reported demonstrate conclusively that in the pituitary preparations used the pressor and oxytocic activities possess different electrophoretic migration rates.

Since the activities migrate at different rates, it is obvious that they are physiological manifestations of different chemical entities present in the preparations electrolyzed.

The results indicate that the rate of migration of the pressor principle towards the cathode cell under the experimental conditions described is approximately 6 times that of the oxytocic principle.

Under the conditions described, neither pressor nor oxytocic activity is destroyed during electrophoresis in periods up to 6 days. After 6 days loss of both activities occurs. Possible factors contributing to this loss have been suggested.

Extension of these findings to routine purification of these principles in a preparative way has been indicated.

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## VITAMIN A AND TUMOR MITOCHONDRIA

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Recently one of the authors (1) showed the effect of a carcinogenic compound, 1, 2, 5, 6-dibenzanthracene, on the vitamin A and total lipid of hepatic mitochondria. The results demonstrated the vitamin A-depleting action of the hydrocarbon on these cytoplasmic structures. A natural inference would be that administration of the vitamin might help in combating this. The possibility proved unlikely, for parenteral injections of large amounts did not alter the results produced by dibenzanthracene. It was apparent that the mitochondria under the experimental conditions observed were not able to store vitamin A even though abundantly supplied. It was, therefore, determined to test the ability of malignant tumors to take up and utilize vitamin A in their mitochondria and compare this with the storage capacity of the hepatic structures in the tumor animal. Because of previous results (1) it was thought to be of interest to study the total lipid of hepatic and tumor mitochondria as contrasted with that of normal liver.

### EXPERIMENTAL

The Flexner-Jobling rat carcinoma and the R-39 rat sarcoma were chosen for analysis. The procedure involved transplantation of the tumors into a number of animals. At various intervals and depending on the size of the tumors these were removed as well as the livers. The method of separation and analysis of mitochondria was carried out as described before (1). A series of rats of the same strain and age was used for control. These were used to secure the normal values which obtain in the livers of non-tumorous animals. All were fed a diet of Purina Dog Chow which proved adequate for maintenance of health and al-

lowed regular gain in weight under normal conditions. It was an excellent source of vitamin A, as shown later on by the liver content.

### RESULTS

The tumor cell mitochondria of both Flexner-Jobling rat carcinoma and R-39 sarcoma showed no vitamin A. This was so in spite of the fact that the hepatic mitochondria of both types of tumor animals had an abundant store of the vitamin, being in fact of the same order as that found in the corresponding struc-

TABLE I  
*Vitamin A and Total Lipid of Tumor and Hepatic Mitochondria*

	Minimum and maximum values observed			
	Vitamin A per 100 mg. tumor mitochondrial lipid*	Vitamin A per 100 mg. liver mitochondrial lipid*	Total lipid in liver mitochondria	Total lipid in tumor mitochondria
	units	units	per cent	per cent
Flexner-Jobling rat carcinoma.....	0	1008-6400	25.1-33.6	23.5-34.1
Mean of 25 rats.....	0	2823	30.1	29.1
R-39 rat sarcoma.....	0	1073-2867	28.6-32.0	10.2-19.6
Mean of 25 rats.....	0	1859	31.2	16.6
Control.....		1059-6772	24.0-32.8	
Mean of 25 rats.....		2133	28.3	

\* These values were determined as in previous work (1) by comparison with a biologically standardized halibut liver oil containing 32,000 u.s.p. units of vitamin A per gm.

tures of normal animals. The weights of the tumors varied from 2.0 gm. to as large as 22.4 gm. Table I gives the minimum and maximum results with an average of each series of animals. The sarcoma rats showed a lower average value for vitamin A in the hepatic mitochondria, but the average as well as each individual result was not below the minimum normal.

The total lipid of hepatic mitochondria of the tumor and control rats was the same. The total lipid of sarcoma mitochondria, although large, was less than in carcinoma, probably owing to the difference in tissue origin. However, in spite of a fairly large total lipid in all tumors the vitamin was absent in every instance.

This confirms the previous work (1) where it was found that a high lipid value for mitochondria does not necessarily result in a large vitamin A content. It seems that simple solution of the fat-soluble vitamin in the mitochondrial lipids will not explain the observed facts. These results and others (2, 3) point rather to a chemical combination of the vitamin with some other constituent. Moreover, this process does not seem to take place in the tumor mitochondria even with an abundant supply present in the host. It is interesting to note at this point that Ronsse (4) and Dittmar (5) who studied the effect of vitamin A on tumor growth came to the conclusion that it had no inhibiting action. Lustig and Wachtel (6) thought its use had an inhibiting effect on certain neoplasms. This was obtainable only at the risk of hypervitaminosis. Sure, Buchanan, and Thatcher (7) with similar massive doses were not able to demonstrate regression or inhibition. Our results with the two types of tumor used served to show that merely increasing the available vitamin A intake can have little effect on the growth processes of the tumor cell.

#### SUMMARY

1. Vitamin A was found to be absent in tumor cell mitochondria even in the presence of an abundant supply in the liver.
2. The vitamin A content and the total lipid of hepatic mitochondria in the tumor animals were normal.
3. The total lipid of tumor mitochondria showed some variation. In the case of sarcoma it was less than in carcinoma.

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# THE RED PIGMENT OF THE ROOT OF THE BEET (BETA VULGARIS)\*

## I. THE PREPARATION OF BETANIN

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(Received for publication, November 24, 1937)

Betanin, the conspicuous red pigment of the root of the beet, was first isolated in 1918 by Schudel (1) and has been recently studied by Ainley and Robinson (2). It has been classified as an anthocyanin, related to the pigments of the petals of many flowering plants, but differs from typical anthocyanins in that it contains nitrogen. The pigment is a somewhat unstable substance and great difficulty has been experienced in preparing pure specimens; the yields reported are small.

Because of the possible significance of this pigment in the study of the genetics of the beet, and also because the intensity of coloration of the root is of importance in determining the value and use of this crop, a study of betanin is being made at this Station. No information with respect to the light absorption of the pigment was available in the literature but, if preparations of reproducible physical properties could be secured, it seemed probable that a simple colorimetric method to determine the quantity in the tissue might be developed, of sufficient accuracy to have significance for genetic and agricultural studies. The present communication describes a procedure for the preparation of samples of pigment that have a constant extinction coefficient. The method is based on the observation that a precipitate that contains most of the pigment separates when an acid alcohol extract of the dried root tissue is neutralized with lithium hydroxide. The concentrate so

\* A brief report of this investigation was given at the meeting of the American Chemical Society at Rochester, New York, September 10, 1937.



obtained, after dehydration, contains approximately 16 per cent of pigment. Purification is effected by precipitation with lead acetate and subsequent separation in indistinctly crystalline form from acidified aqueous solution, or by precipitation from alcohol solution with peroxide-free ether.

†

## EXPERIMENTAL

*Preparation of Pigment Concentrate*—Fresh beet-roots are grated; the pulp is dried in a thin layer at 80° in a ventilated oven, and is ground in a meat grinder to a coarse powder. This is treated, in lots of 500 gm., three or four times successively with 1 liter of boiling 95 per cent alcohol, being filtered each time. Yellow pigments and other troublesome contaminants are thereby removed. The residue is dried and ground in a ball mill to a fine powder. 100 gm. of the powder are suspended in 150 ml. of 95 per cent ethyl alcohol and 15 ml. of a 20 per cent solution of hydrogen chloride in ethyl alcohol (20 gm. in 100 ml.) are added. After the mixture has been stirred for 20 minutes at room temperature, 350 ml. more of alcohol are added and stirring is continued for 30 minutes. The solution, which contains most of the red pigment, is then drawn off with suction through a tube to the end of which a small Buchner funnel covered with filter cloth is attached. A second extraction with 150 ml. of alcohol, 10 ml. of alcoholic hydrogen chloride, and, later, 300 ml. of alcohol is carried out in the same manner, and two more extractions with, respectively, 150, 5, and 350 ml. of reagents. The combined extracts are filtered and neutralized to moistened Congo red paper with 9 per cent aqueous solution of lithium hydroxide added dropwise with continuous mechanical stirring. The precipitate is allowed to settle, and the supernatant alcohol is siphoned off. The insoluble material is washed several times by decantation with 95 per cent alcohol and is then suspended in acetone and filtered. The product is dried at 60–70° in the ventilated oven and is finally pulverized and preserved in closed containers. The yield is ordinarily between 5 and 8 gm., depending upon the variety of beets used.

*Lead Acetate Precipitation*—5 gm. of the concentrate are dissolved in 300 ml. of water and filtered, and 30 per cent lead acetate solution is slowly added until a centrifuged test sample shows no additional precipitation (40 to 45 ml. are usually required). The

ing surfaces and the preparation may be regarded as indistinctly crystalline. The substance is hygroscopic and must be carefully protected against moisture.

The aqueous solution is purplish red and, at a concentration of 0.005 mg. per ml. at pH 5.2, has an extinction coefficient of 0.398 when measured in a 1 cm. cell with a Zeiss spectrophotometer with Filter S-53. This extinction coefficient was not increased by attempts at further purification. The conditions under which this measurement is made are fully described in Paper II of this series (4).

Chromatographic analysis on tricalcium phosphate revealed no inhomogeneity, and, after elution with acid alcohol and precipitation with ether, the extinction coefficient was unchanged.

On being dried *in vacuo* over phosphorus pentoxide at 100°, the product lost the equivalent of slightly more than 3 molecules of water. It retained a little chlorine, some of which appeared to be lead chloride.

An aqueous solution becomes slightly more red when sodium carbonate is added, and slightly bluer when acidified. The color is immediately discharged to a yellow by sodium hydroxide, more slowly to a brown by ammonia. The solution in cold dilute hydrochloric acid changes but little on standing but, on being heated, soon becomes blue-violet. With lead acetate, the color becomes more yellow and a red precipitate separates, leaving the solution still yellowish red. The pigment cannot be recovered unchanged from this precipitate. If a soluble phosphate is also present, however, the addition of lead acetate removes nearly all of the pigment on the precipitate, leaving the supernatant fluid pale pink, and the pigment can be regained from the precipitate apparently unchanged when this is treated with hydrochloric acid. It is upon this behavior that the success of the method of purification of the crude concentrate probably depends. The aqueous solution of the pigment gives no distinctive test with ferric chloride, the color changing to yellow-brown. The color is also destroyed by permanganate, and the pigment can be quite accurately titrated to a colorless end-point with this reagent, 8 atoms of oxygen being required for 2 nitrogen atoms in the pigment.

*Sugar Determination*—50 mg. samples of purified pigment, dried at room temperature over phosphorus pentoxide, were heated in

a boiling water bath with 7 ml. of 2 N sulfuric acid for 1, 3, and 5 hours respectively. The hydrolysates were neutralized with barium hydroxide, 5 ml. of 0.2 N oxalic acid were added, and the solutions were made to 200 ml. Analyses for sugar (5) after treatment with Lloyd's reagent gave respectively 29.1, 28.5, and 26 per cent calculated as glucose, the whole of which could be removed in the usual way with a suspension of yeast and is therefore probably glucose. Corrected for 11.5 per cent of water and 2.1 per cent of ash, the highest of these values is equivalent to 33.7 per cent of glucose in the product. The Lloyd's reagent removes a small amount of decomposition products that reduce the sugar reagent but which are not removed by yeast.

The values found for sugar are low, the formula deduced from the elementary analysis requiring approximately 38 per cent. The progressive loss during hydrolysis was accompanied by gradual destruction of the color of the pigment, this being entirely replaced by orange at the end of 5 hours of hydrolysis.

Additional evidence that the sugar is probably glucose was obtained by the isolation of phenylglucosazone. To this end, 1.0 gm. of pigment was heated for 4 hours with 70 ml. of 4 N sulfuric acid at 100°; the reagent was removed with barium hydroxide and decomposition products were precipitated by the addition of 2 volumes of alcohol. After concentration to small volume and adjustment of the reaction with sodium acetate and acetic acid, excess of phenylhydrazine was added and the solution was heated to 100° for 1 hour. The precipitate was removed, washed, and recrystallized from hot aqueous alcohol; m.p. 204–206° (uncorrected). Mixed with an equal amount of authentic phenylglucosazone, m.p. 206° (uncorrected); yield, 0.164 gm.

*Composition of Pigment*—Microanalyses of the purest specimen of the pigment obtained are given in Table I.<sup>1</sup> The samples, previously dried at room temperature, when dried at 100° over phosphorus pentoxide, lost 11.57, 11.33, and 11.58 per cent of moisture in three analyses. The inorganic residue was 1.71 and 2.12 per cent in two cases and a macroanalysis gave 2.50 per cent; the average of these figures is 2.11 per cent. The low chlorine content recalls the experience of Ainley and Robinson who state that the

<sup>1</sup> We are indebted to Professor H. T. Clarke and Mr. Saschek for these determinations.

hydrochloric acid in the preparation of beet pigment they studied may be in part replaced by water. This circumstance, together with the occurrence of lead chloride in the ash of our preparations, introduces some difficulty into the interpretation of the analysis. It is improbable that the value assigned to the ash has great significance because of the volatility of lead chloride. However, if it be assumed that the whole of the ash consisted of lead chloride, a value for the organic chlorine can be calculated, and this, together with the corrected values for the other elements, is given in Column 2. The ratio between carbon and nitrogen atoms is either 20:2 or 21:2. The choice of the latter ratio is necessary if the product is to be formulated as a glucoside of a nucleus with 15

TABLE I  
*Composition of Beet Pigment*

The samples were dried over phosphorus pentoxide at 100°. The values are given in per cent.

	Found (1)	Corrected for ash (2)	Calculated for		
			$C_{21}H_{23}N_2O_{10}Cl$ (3)	$C_{21}H_{23}N_2O_{10} \cdot OH$ (4)	$C_{21}H_{23}N_2O_{10}$ (5)
C	52.18	53.31	50.55	52.48	54.53
H	4.77	4.87	4.65	5.04	4.58
N	5.93	6.06	5.62	5.83	6.06
Cl	1.54	1.02	7.10	0.0	0.0
O		34.74	32.08	36.65	34.61
Ash	2.11				

carbon atoms. The ratio of nitrogen to oxygen atoms is 1:5. If it be assumed that the nitrogen value is accurate, the hydrated pigment contains 5.32 per cent of nitrogen, and the ratio between nitrogen atoms and water molecules is 2:3.36. The analysis conforms fairly closely with the theoretical requirements of a substance of the formula  $C_{21}H_{23}N_2O_{10}Cl \cdot 3H_2O$  which, in the course of isolation, has had a considerable part of its chlorine replaced by water and this product has in turn been partially dehydrated. This formula corresponds to a glycoside of a nitrogenous anthocyanidin,  $C_{15}H_{13}N_2O_5Cl \cdot C_6H_{10}O_5 \cdot 3H_2O$ . If it be assumed that four hydroxyl and two amino groups are present, the requirements of the structural formula are met.

It must be pointed out, however, that the classification of this pigment as a nitrogenous anthocyanidin derivative is still speculative. Typical anthocyanins on acid hydrolysis yield insoluble and relatively stable, intensely colored anthocyanidins, and phloroglucinol is invariably found among the products of alkali decomposition. The beet pigment, on the other hand, is extensively decomposed by acid hydrolysis. Phloroglucinol was not detected among the products of alkali decomposition either by Schudel, by Ainley and Robinson, or in this laboratory.

No success has as yet attended our efforts to establish the nature of the nitrogen in the beet pigment. The compound is instantly decolorized by the nitrous acid reagent in the Van Slyke apparatus at room temperature, and gas equivalent to approximately 18 per cent of the nitrogen present is liberated under the usual conditions. A similar value is obtained both before and after mild acid hydrolysis. It is not certain, however, that the gas is derived from amino groups; Stuart (6) has reported that phenolic substances yield gas in the Van Slyke apparatus, an observation that has been confirmed in this laboratory. Phloroglucinol, for example, behaves as if it contained about 8 per cent of amino nitrogen. Accordingly, no definite conclusion can be drawn from this test of the beet pigment save that the nitrogen is probably not primary aliphatic amino nitrogen. Neither the products of hot alkali decomposition nor of severe acid hydrolysis gave a ninhydrin reaction; an aliphatic  $\alpha$ -amino acid configuration is therefore absent.

When subjected to the action of nitrous acid at 0°, the beet pigment is not decolorized and gives no indication of the evolution of gas. Furthermore, there is no evidence of utilization of nitrous acid. This might be expected to occur through diazotization of aromatic amino groups, if such were present; the failure to observe it may indicate a high degree of inactivity, possibly due to the structure of the compound. In any case, whatever the form of nitrogen may be, the groups are extraordinarily weakly basic; the compound, even though separated from an acid solvent, retains much less than the theoretical quantity of chlorine required by the oxonium group alone.

The nitrogen of the pigment is only incompletely oxidized to ammonia by the Kjeldahl method; values of 90 to 95 per cent of the nitrogen indicated by the micro-Dumas method were secured. If the nitrogen atoms are present as amino groups, this behavior

would not be anticipated, and a hint is provided that part of the nitrogen may occur in a ring structure.

*The Betanidin of Ainley and Robinson*—The pigment isolated by Ainley and Robinson was prepared from beet tissue that had been suspended in water and allowed to undergo spontaneous fermentation. The subsequent steps involved extraction with isoamyl alcohol, the pigment being thrown out of solution in this solvent by petroleum ether and collected in concentrated solution in water. Purification was effected with solvents, the solid being finally isolated from dilute hydrochloric acid. Their product contained considerable chlorine, although less than that required by theory for 1 equivalent of hydrochloric acid.

TABLE II  
*Analysis of Beet Pigment*

Carbon, hydrogen, nitrogen, and glucose were recalculated on a chlorine-, water-, and ash-free basis; chlorine and ash were calculated on material dried at 100°. The values are given in per cent.

	This paper	Ainley and Robinson	Schudel
C	54.2	56.6	55.2
H	4.95	5.41	4.97
N	6.16	6.68	8.57
Glucose	33.7		28.4
OCH <sub>3</sub>		0.0	3.95
Cl	1.54	5.0	0.78, 6.93
Ash	2.11	3.0	

The preparation was regarded as an anthocyanidin chloride produced by enzymatic hydrolysis of the original anthocyanin during the first step of the preparation. The analysis corresponded with the formula  $C_{20}H_{19-23}O_7N_2Cl \cdot 3H_2O$  in which about 30 per cent of the hydrogen chloride had been replaced by water, and they advanced the suggestion that the constitution of the substance may be represented as a pentahydroxyflavylium nucleus condensed with ornithine by means of one of the basic groups of this substance. Such a structure is in agreement with the analytical findings and many of the chemical properties. No observation with respect to the presence of sugar was recorded, although Price and Robinson (7) mention that a pigment obtained from beet tissue by an alumina absorption method was a glycoside.

Because of the difference in chlorine content, it is difficult to

compare their product with the one we have obtained. Table II, however, shows their data recalculated on a basis free from chlorine, compared with ours, likewise recalculated. The figures given by Schudel are also included.

Ainley and Robinson's data indicate a carbon to nitrogen atomic ratio of 20:2, but are in closer agreement with a compound that contains 8 oxygen atoms than with one that contains 7. The general properties of their product correspond in almost every detail with those we have observed with the exception that our product is a glycoside.

Interpretation of the analytical results of Schudel is difficult, probably in part because carbon and hydrogen were determined on a sample of low chlorine content, the nitrogen on one that contained nearly the theoretical amount of chlorine for 1 atom. Furthermore, he did not report the ash content, and it seems possible that his high chlorine specimen may have been impure, because it evidently yielded an appreciable quantity of methoxyl; the nitrogen value also appears to be high. His product was, like ours, definitely a glycoside.

#### SUMMARY

The preparation of the red pigment of the root of the beet in a form that is substantially pure and homogeneous is described. The method is simple and expeditious and the product has a constant extinction coefficient. It is a glucoside of a nitrogenous nucleus that probably contains 15 carbon atoms and appears to be related to the anthocyanidins. The form of combination of the nitrogen atoms is not known, but there is probably no aliphatic amino group. The evidence for an aromatic amino group is inconclusive; the presence of ring nitrogen is possible.

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# THE RED PIGMENT OF THE ROOT OF THE BEET (BETA VULGARIS)\*

## II. A METHOD TO DETERMINE BETANIN

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The intensity of the red pigmentation of the root of the beet has an important relationship to the commercial value and use of this crop both in the market gardening and canning industries. No simple method has hitherto been suggested, however, by which the intensity of the color can be expressed in quantitative terms. A method whereby the actual pigment concentration could be ascertained with reasonable accuracy would have immediate application to the study of the genetic and environmental factors that influence the color in this species.

The isolation of this pigment in substantially pure form, described in Paper I of this series (1), placed in our hands a reproducible material the physical properties of which can be employed for analytical purposes. Although subsequent investigation may reveal methods whereby a more highly purified preparation can be secured, its physical constants will be related in a definite manner with those of our present preparations, and suitable correction factors can be computed and applied.

*Light Absorption of Betanin*—A stock solution of the purified pigment (1) is prepared by transferring 10 mg. of the finely powdered dry material, accurately but rapidly weighed, to a small mortar in which it is thoroughly ground with 8 to 10 drops of water; 10 ml. of succinic acid-borate buffer of pH 5.2 are added; the solid is triturated, and the clear solution decanted. Undissolved

\* A brief report of this investigation was given at the meeting of the American Chemical Society at Rochester, New York, September 10, 1937.



particles are similarly treated with successive small quantities of buffer solution until complete solution is secured. The solution is then made to 100 ml. with water; it is stable for at least 1 hour. Dilutions of from 1:10 to 1:50 made with the buffer solution are suitable for spectrophotometric measurements, but a dilution of 1:20 is taken as an arbitrary standard. This represents a concentration of 0.005 mg. per ml.

An approximate light transmission curve was obtained by observation of the standard solution with the color filters of a Zeiss spectrophotometer. Table I shows that maximum absorption is obtained at the effective wave-length  $530\text{ m}\mu$ , and the No. S-53 filter was accordingly chosen for subsequent measurements. It is not ideal and can be employed with case only with fairly dilute solu-

TABLE I

*Light Transmission of Betanin*

Concentration, 0.005 mg. per ml.; pH 5.2; 1 cm. cell.

Filter No.	Effective wave-length	Transmission
	<i>mμ</i>	<i>per cent</i>
S-61	617	100
S-57	572	64.7
S-53	530	40.7
S-50	494	58.6
S-47	463	80.6
S-43	435	90.0

tions. Satisfactory reproducibility is, however, obtained with concentrations between 0.001 and 0.01 mg. per ml.

*Stability of Betanin*—The stability of the standard solution with respect to reaction and time was studied by means of observations on solutions obtained by dilution of 1 ml. of the stock solution to 20 ml. with appropriate buffer solutions. The extinction coefficient obtained immediately at pH 5.2 was taken as standard, and the observations in Table II are expressed as percentages of this value. It is clear that at a reaction in the neighborhood of pH 5 the pigment is stable for a period that permits useful results to be obtained.

*Extinction Coefficient*—The validity of a colorimetric method depends upon the precision with which Beer's dilution law is fol-

lowed. Table III shows data on the extinction coefficients of solutions of the beet pigment of different concentrations. The agreement between the ratios of the concentration and of the extinction coefficients is sufficiently close to insure that Beer's law is applicable within the concentration range studied.

The value for the extinction coefficient of the arbitrary standard solution of concentration 0.005 mg. per ml. rests upon thirty deter-

TABLE II  
*Stability of Color of Betanin at Various Reactions*

Filter, S-53; concentration, 0.005 mg. per ml.; 1 cm. cell. The data are expressed as per cent of the extinction coefficient at pH 5 and 0 time.

pH	0 hr.	1.7 hrs.	3.3 hrs.	7.3 hrs.
1.0	85.7	45.2	39.6	30.0
3.0	96.7	88.5	78.1	68.6
4.0	98.7	94.0	91.0	81.9
5.0	100.0	96.7	94.0	90.8
6.0	100.3	96.7	93.0	80.3
7.0	98.1	77.2	73.2	59.5

TABLE III  
*Relationship between Concentration and Extinction Coefficient of Betanin*  
Filter S-53; solutions at pH 5.2.

Betanin concentration	Cell length	Extinction coefficient	Ratio of concentration	Ratio of extinction coefficient
<i>mg. per ml.</i>	<i>cm.</i>	<i>K</i>		
0.010	0.5	0.807	2.0	2.03
0.0067	0.5	0.533	1.37	1.34
0.005	1.0	0.398	1.00	1.00
0.004	1.0	0.314	0.80	0.786
0.002	2.0	0.158	0.40	0.395
0.001	3.0	0.079	0.20	0.198

minations made upon two of the purest specimens obtained and on a mixture of these. The mean value was 0.3974 and the standard error 0.0010. Because somewhat greater weight may properly be attached to the higher values, the value 0.398 was provisionally adopted for use in the quantitative determination of the pigment. Should later work indicate a somewhat higher value for more completely purified pigment, a correction of the data in this paper can easily be applied. The mean value of sixteen dif-

ferent preparations, some of which were known to be less pure than others, was  $0.389 \pm 0.003$ ; individual values ranged from 0.366 to 0.408.

*Determination of Betanin in Beet-Root Tissue*—The method of determination developed from the foregoing data can be applied to the tissue from a single root or to larger samples. The weighed sample is carefully grated to a pulp which is spread in a thin layer on a pan and dried in a ventilated oven at  $80^{\circ}$ . The dry product is weighed, roughly ground in a mortar, and is then ground in a ball mill<sup>1</sup> until reduced to a fine powder. It is usually impossible to remove the sample quantitatively from the mill, but the relationship between the analysis of the ground product and the origi-

TABLE IV  
*Betanin Content of Beet-Root Tissue*

The data are expressed as per cent of dry weight.

Variety	Method	Betanin
Detroit dark red (dark strain)	Water extract	1.19
		1.20
	2 preliminary cold alcohol extractions	1.14
		1.18
		1.16
	hot	1.14
Improved blood turnip	Water extract	0.34
Crosby's Egyptian		0.15
Good-for-all		0.47
Sutton's pineapple		0.46

nal moist or dry tissue is easily calculated. Difficulties encountered with grinding are usually traceable to incomplete drying.

A sample of 0.050 or 0.100 gm. of the powdered tissue is weighed into a  $200 \times 25$  mm. test-tube graduated at 50 ml. The sample is moistened with a few drops of water, triturated with a rod, and 20 ml. of succinic acid-borate buffer of pH 5.2 are added. The solution is diluted to the mark with water; the tube is stoppered and is shaken vigorously for a few minutes and centrifuged. The clear extract is decanted through a plug of glass wool in a dry funnel. The percentage transmission of the filtrate is read at the Zeiss spectrophotometer with Filter S-53 within half an hour, and the

<sup>1</sup> A suitable mill is described by Pucher and Vickery (2).

extinction coefficient  $K$  is calculated. The relation  $(K/0.398) \times 0.005$  gives the number of mg. of betanin in 1 ml. of the extract. If the concentration of the extract is greater than 0.01 mg. per ml., an appropriate dilution of the extract is made with buffer solution as diluent, and the transmission is again observed.

Water extracts of the dried root tissue are usually satisfactory. Occasionally, however, a turbidity is encountered that cannot be centrifuged. In this case, a preliminary extraction of the sample with two 5 ml. quantities of absolute alcohol is made. The tubes are heated in a water bath at 70–80° for 5 minutes, cooled, and centrifuged. The alcohol extracts are discarded and the analysis is continued on the moist residue. Table IV gives data that illustrate the effect of preliminary alcohol extractions of the dry tissue, and the reproducibility of duplicate determinations in which exceptionally highly pigmented tissue was employed. Typical values for a few other varieties of beets are also shown. It is clear that the preliminary extractions with alcohol which remove yellow pigments have little effect upon the values for betanin obtained with the color filter recommended.

Color differences in beets have been attributed to differences in genetic constitution (3), but environmental factors may also play a part in determining how the genetic factors are expressed. The present method is to be employed in studies of these points.

#### SUMMARY

A simple method to determine the red pigment content of beet-root tissue is described. The light transmission of a water extract of the dry tissue is determined with color Filter S-53 of a Zeiss spectrophotometer. The concentration in terms of the purified isolated pigment, which has an extinction coefficient of 0.398 at a concentration of 0.005 mg. per ml., is then calculated. The method can be applied to the study of the genetic and environmental factors that influence the quantities of pigment in different varieties and inbred lines of beets.

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# THE REDUCTION OF AMINOSORBITOL HYDROCHLORIDE WITH HYDRIODIC ACID

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Inasmuch as attempts at the reduction of glucosaminic acid to optically active norleucine have met with great difficulties, it was decided to select a different approach to the solution of the problem of the configuration of glucosamine; namely, the conversion of glucosamine into 2-aminopentahydroxyhexane<sup>1</sup> (2-aminosorbitol) in the hope that by reduction with hydriodic acid the hydroxyamine would be converted into 2-aminoheptane. Thus a direct answer to the configuration of *d*-glucosamine would be furnished, since the configuration of 2-aminoheptane has been correlated by Levene and Mardashew<sup>2</sup> to that of 2-aminocaproic acid. The work has not yet been completed but the results so far obtained are reported here in view of the activities of other laboratories on the question of the configuration of *d*-glucosamine.

The product so far obtained by reduction with aqueous hydriodic acid has the composition of 2-aminoheptene oxide; on acetylation it forms a monoacetyl derivative which no longer possesses a free amino group but on deacetylation the amino group becomes free again. This acetylated substance possesses no ethylenic linkage and is not a double molecule formed by union of 2 monohydroxyaminoheptane molecules, since a molecular weight of 166 was obtained by the Rast method. The composition of the substance, therefore, is  $C_7H_{15}ON \cdot HCl$ .

Further reduction of this substance is now in progress. Also, other methods of arriving at the configuration of the 2-aminopentahydroxyhexane are now in progress.

<sup>1</sup> Levene, P. A., and Christman, C. C., *J. Biol. Chem.*, **120**, 575 (1937).

<sup>2</sup> Levene, P. A., and Mardashew, S., *J. Biol. Chem.*, **117**, 707 (1937).

It may be mentioned that reduction of the hexaacetate of 2-aminopentahydroxyhexane with hydriodic acid in glacial acetic acid resulted in a 2-aminomonohydroxyhexane directly and its acetate by acetylation of the substance from the mother liquor. Unfortunately the yield was so small that the experiment could not be repeated with sufficiently consistent success to warrant further experimentation.

#### EXPERIMENTAL

*Treatment of Aminosorbitol Hydrochloride with Hydriodic Acid*—A solution of 5 gm. of aminosorbitol hydrochloride<sup>1</sup> in 60 cc. of hydriodic acid (sp. gr. = 1.70) was heated in a sealed tube during 5 hours at a temperature of 125°. The furnace and tube were allowed to cool for 18 hours, after which the tube was opened and the contents diluted with water to 180 cc.

The solution, containing much free iodine, was evaporated under diminished pressure to about 75 cc. and then diluted to 1000 cc. with water. The major portion of the free iodine and hydriodic acid was removed by adding lead carbonate and then filtering off the lead salts. (In this and in all the following operations the precipitates were shaken with water and then filtered, the filtrates being combined with the original filtrate.) The remainder of the iodine and hydriodic acid was removed with silver carbonate in the presence of free sulfuric acid. After removal of all the silver and lead ions with hydrogen sulfide, the solution was made alkaline and steam-distilled into dilute hydrochloric acid. The distillation was continued until the distillates were no longer alkaline to red litmus paper. The acid solution of the distillate was evaporated under diminished pressure to a sirup, which was dried by repeated addition and concentration of benzene and absolute ethyl alcohol. The dried sirup was dissolved in absolute ethanol, filtered from the ammonium chloride, and an equal volume of ether added to the filtrate. More ammonium chloride separated out after standing in the refrigerator overnight and this was also filtered off. The sirup obtained by concentration of this filtrate was used in the next experiment.

*Preparation of Crystalline Chloroplatinate from Reaction Product Obtained by Treatment of Aminosorbitol Hydrochloride with Hydriodic Acid*—The dried sirup from the previous experiment was

dissolved in a small volume of absolute ethanol and an excess of chloroplatinic acid added. Ammonium chloroplatinate, if present, was removed by filtration and the filtrate concentrated to half its volume in a vacuum desiccator.

In this way a crop of crystals was obtained which were recrystallized by dissolving in warm absolute ethanol, filtering, and then allowing the filtrate to evaporate spontaneously in a desiccator. The average yield obtained in several experiments was about 1.5 gm. (from 5 gm. of aminosorbitol hydrochloride).

This chloroplatinate had a composition which agreed fairly well with that calculated for the chloroplatinate of an aminohexene oxide. However, the analytical data varied with different preparations (indicating the presence of other material). By repeated careful recrystallization a product was obtained which had the following composition.

4.780 mg. substance: 3.902 mg.  $\text{CO}_2$  and 1.895 mg.  $\text{H}_2\text{O}$

$\text{C}_{12}\text{H}_{28}\text{O}_2\text{N}_2\text{PtCl}_6$ . Calculated. C 22.48, H 4.5

Found. " 22.26, " 4.4

*Preparation of 2-Aminohexene Oxide Hydrochloride*—9 gm. of the once recrystallized chloroplatinate (from the previous experiment) were dissolved in 100 cc. of warm water. Hydrogen sulfide was passed into the solution for 3 hours and the mixture allowed to stand overnight in the presence of hydrogen sulfide. The platinum sulfide was removed by filtration and well washed with warm water. The combined filtrates were concentrated to 25 cc., treated with charcoal, and then filtered.

This filtrate was concentrated to a dry crystalline mass which was dried further by frequent addition and evaporation of benzene and absolute alcohol. The product was obtained in the pure state by recrystallizing from a small volume of absolute ethanol. Yield 2.0 gm. All the mother liquors were concentrated to dryness. Yield 1.4 gm.

The pure substance had a melting point of 217–218° and a specific rotation of  $[\alpha]_D^{25} = \frac{-0.30^\circ \times 100}{2 \times 2.54} = -5.9^\circ$  (in absolute ethanol). It is soluble in alcohol and water but practically insoluble in ether, acetone, chloroform, and pentane.

The compound had a composition agreeing with that of 2-aminohexene oxide hydrochloride.



3.981 mg. substance: 6.914 mg. CO<sub>2</sub> and 3.330 mg. H<sub>2</sub>O

6.078 " " : 0.498 cc. N<sub>2</sub> (748 mm. at 28°)

7.478 " " : 4.90 cc. 0.01 N AgNO<sub>3</sub>

C<sub>6</sub>H<sub>13</sub>ON·HCl. Calculated. C 47.49, H 9.3, N 9.20, Cl 23.08

Found. " 47.36, " 9.36, " 9.14, " 23.26

*Attempted Catalytic Hydrogenation of 2-Aminohexene Oxide Hydrochloride*—200 mg. of crystalline 2-aminohexene oxide hydrochloride dissolved in 30 cc. of absolute ethanol were shaken with Adams' catalyst and hydrogen for several hours. The catalyst was removed by filtration and the filtrate concentrated to about 4 cc. Unchanged starting material was isolated from this solution by the addition of ether, as indicated by the melting point (217–218°) and analysis, thus indicating the presence of an oxygen ring and the absence of any double bonds.

*Acetylation of 2-Aminohexene Oxide Hydrochloride*—Pure 2-aminohexene oxide hydrochloride (0.6 gm.) was refluxed for 2 hours with 10 cc. of acetic anhydride and 1 gm. of freshly fused sodium acetate. The mixture was allowed to stand overnight at room temperature and then concentrated to dryness under reduced pressure. All traces of acetic anhydride and acetic acid were removed by repeated concentrations with the addition of small volumes of benzene.

The crystalline mass was now treated with 15 cc. of chloroform and filtered. The filtrate was dried with sodium sulfate and concentrated to a dry mass of crystals. The product was recrystallized by dissolving in an acetone-ether mixture and then adding a small volume of pentane. Yield 0.55 gm. The substance was pure after three such recrystallizations and had a melting point of 142–143°. It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.29^\circ \times 100}{2 \times 3.56} = +4.1^\circ \quad (\text{in absolute ethanol})$$

The substance showed no perceptible rotation in chloroform solution. It is soluble in acetone, ether, chloroform, water, and benzene but is practically insoluble in pentane.

The material had a composition agreeing with that of an N-acetylaminohexene oxide. It contained no free amino nitrogen and a Rast molecular weight determination gave a value of 166, which is in accord with the calculated value of 157.

4.594 mg. substance: 10.310 mg. CO<sub>2</sub> and 3.897 mg. H<sub>2</sub>O

5.994 " " : 0.455 cc. N<sub>2</sub> (759 mm. at 25°)

5.402 " " : 3.371 " 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>\*

C<sub>9</sub>H<sub>13</sub>O<sub>2</sub>N. Calculated. C 61.14, H 9.6, N 8.9, COCH<sub>3</sub>, 27.39

Found. " 61.20, " 9.5, " 8.7, " 26.83\*

*Action of Hydrogen Iodide in Glacial Acetic Acid on Aminosorbitol Hydrochloride*—Two sealed tubes, each containing 5 gm. of aminosorbitol hydrochloride, 0.5 gm. of phosphonium iodide, and 40 cc. of a solution composed of equal parts by weight of dry hydrogen iodide and dry glacial acetic acid, were heated at 125° for 5 hours. The materials were united and the product isolated in the same way as in the previous experiment with hydriodic acid.

The dried, steam-distilled sirup was treated with chloroplatinic acid but only about 0.2 gm. of crystalline chloroplatinate was obtained.

*Action of Hydrogen Iodide in Glacial Acetic Acid on 2-Aminosorbitol Hexaacetate*<sup>1</sup>—To 6 gm. of pure 2-aminosorbitol hexaacetate were added 25 cc. of a solution composed of equal parts by weight of dry hydrogen iodide and glacial acetic acid, and the mixture sealed in a bomb tube. After heating at 125° during 4 hours and then cooling in the furnace for 12 hours, the tube was opened and the contents diluted with 150 cc. of water.

Sulfur dioxide was passed into the solution until all the free iodine had been consumed. The excess sulfur dioxide was removed by aspirating with air and the sulfuric acid was removed quantitatively by the addition of barium hydroxide solution. This solution was then concentrated to dryness under diminished pressure at 40°, and the residue was dissolved in 100 cc. of methyl alcohol and reduced with hydrogen and Raney's catalyst.

When the reduction was complete, the catalyst was removed by filtration and the methyl alcohol solution steam-distilled in alkaline solution until the final distillate was no longer alkaline to red litmus. The distillate was collected in a solution of hydrochloric acid in order to prevent the loss of the volatile free base.

\* This substance has the acetyl group bound so firmly to the nitrogen that the time of digestion in the acetyl determination had to be increased (from 3) to 6 hours. This is the first compound which Dr. Elek has found to require more than 3 hours for completion of the deacetylation.

The acid distillates were then concentrated to dryness under reduced pressure at 40° and a small amount of crystalline material was obtained by dissolving the partially crystalline residue in acetone and then adding ether. Yield 0.05 gm. This was recrystallized twice from acetone and then melted at 86–88°.

The substance had the following composition.

4.299 mg. substance:	7.302 mg. CO <sub>2</sub> and 3.995 mg. H <sub>2</sub> O; 2% ash
5.120 " " "	: 0.394 cc. N <sub>2</sub> (760 mm. at 27°)
C <sub>6</sub> H <sup>18</sup> ON·HCl. Calculated.	C 46.9, H 10.45, N 9.12
Found (ash-free).	" 47.1, " 10.5, " 8.92

The acetone-ether mother liquors were combined and evaporated to dryness. The dry sirup was refluxed for 1 hour with a mixture of anhydrous sodium acetate and acetic anhydride, and then kept at room temperature for 1 day. This mixture was now concentrated to dryness and the product extracted with chloroform. The chloroform extract was washed with three small portions of water and then dried with anhydrous sodium sulfate.

The product partly crystallized after the removal of the chloroform and was completely crystallized from ether and pentane. Yield 0.25 gm. After one more recrystallization from ether and pentane the substance melted at 77–78° and had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+1.62^\circ \times 100}{1 \times 4.08} = +39.7^\circ \quad (\text{in chloroform})$$

This material had the following composition.

4.416 mg. substance:	9.590 mg. CO <sub>2</sub> and 3.790 mg. H <sub>2</sub> O
6.420 " " "	: 6.36 cc. 0.01 N Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>
C <sub>10</sub> H <sub>19</sub> O <sub>3</sub> N. Calculated.	C 59.9, H 9.5, COCH <sub>3</sub> 42.8
Found.	" 59.2, " 9.6, " 42.6

## THE REDUCTION OF GLUCOSAMINIC ACID WITH HYDROGEN IODIDE IN GLACIAL ACETIC ACID

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Although the configuration of glucosamine formulated by one of the present authors (P.A.L.) has been substantiated in the last few years by Bergmann *et al.*<sup>1</sup> and more recently by Karrer and Meyer,<sup>2</sup> yet all the evidence thus far furnished has been based on indirect evidence and, in addition, is contradicted by the conclusion of Neuberg, Wolff, and Niemann<sup>3</sup> reached by the method of classical organic chemistry. However, the experiments of Neuberg could never be duplicated. Levene and Wildman<sup>4</sup> treated every one of the eight *D*-2-aminohexonic acids according to the directions of Neuberg, Wolff, and Niemann<sup>3</sup> but the 2-amino-hydroxy acid obtained by them in the first phase of Neuberg's process was invariably inactive. The attempts of Karrer and Meyer<sup>5</sup> to repeat Neuberg's experiments likewise met with little success.

It seemed that if the formation of the 2-aminohydroxycaproic acid could be accomplished without complete racemization, then its transformation into optically active norleucine would be promising. Optically active 2-aminohydroxycaproic acid has now been obtained from glucosaminic acid by reduction with hydriodic acid dissolved in glacial acetic acid. Great difficulties, however, were encountered in the preparation of pure derivatives

<sup>1</sup> Bergmann, M., Zervas, L., Rinke, H., and Schleich, H., *Z. physiol. Chem.*, **224**, 33 (1934).

<sup>2</sup> Karrer, P., and Meyer, J., *Helv. chim. acta*, **20**, 407 (1937).

<sup>3</sup> Neuberg, C., Wolff, H., and Niemann, W., *Ber. chem. Ges.*, **35**, 4009 (1902).

<sup>4</sup> Levene, P. A., and Wildman, E. A., unpublished work.

<sup>5</sup> Karrer, P., and Meyer, J., *Helv. chim. acta*, **18**, 782 (1935).

of this hydroxy acid, owing to simultaneous formation of a lactone, whose ready formation may be taken as an indication that the hydroxyl group in the hydroxy acid is situated on carbon atom (4). Thus the substance probably has the following structure,  $\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$ .

Efforts to reduce this substance to optically active norleucine are being continued.

#### EXPERIMENTAL

*Action of Hydrogen Iodide in Acetic Acid on Glucosaminic Acid*—Four sealed bomb tubes, each containing 5 gm. of glucosaminic acid (finely powdered), 0.5 gm. of phosphonium iodide, and 30 cc. of a solution composed of equal parts by weight of dry hydrogen iodide and dry glacial acetic acid, were heated at  $125^\circ$  during 4 hours. The tubes were then opened, and the contents removed, combined, and diluted to 1 liter with water. The aqueous solution was now extracted several times with chloroform in order to remove the excess iodine.

In the following operations all the lead and silver precipitates and residues were triturated and washed several times with hot water. These washings were then added to the filtrate from which the precipitate or residue had been removed. All concentrations were performed at  $40\text{--}50^\circ$  under reduced pressure.

The aqueous solution, after chloroform extraction, was treated with washed lead carbonate until nearly free from halogen. The lead precipitate was removed by filtration and the filtrate and washings treated with silver carbonate until free of halogen. The silver residues were then removed by filtration and the silver and lead ions removed from this filtrate by means of hydrogen sulfide.

The solution was now concentrated to 200 cc. and again treated with silver carbonate and hydrogen sulfide. The filtrate was concentrated to a thick sirup which crystallized upon the addition of 200 cc. of absolute ethyl alcohol. The material was removed by filtration, and the filtrate diluted with water and again treated with silver carbonate and hydrogen sulfide. Another crop of crystals was obtained by adding alcohol again to the concentrated filtrate. Total yield 5.6 gm.

The product was recrystallized by dissolving in the minimum amount of cold water and then adding an excess of absolute

ethyl alcohol. In this way 4.9 gm. of material were obtained which had the following rotation.

$$[\alpha]_D^{25} = \frac{-1.70^\circ \times 100}{2 \times 5.186} = -16.4^\circ \text{ (in 20\% hydrochloric acid)}$$

The substance had a composition agreeing with that for monohydroxyaminocaproic acid. \*

4.892 mg. substance: 8.718 mg. CO<sub>2</sub> and 3.789 mg. H<sub>2</sub>O; 0.6% ash  
 23.30 " " : 4.16 cc. N<sub>2</sub> (762 mm. at 25°, Van Slyke)  
 C<sub>6</sub>H<sub>13</sub>O<sub>2</sub>N. Calculated. C 49.00, H 8.9, amino N 9.52  
 Found (ash-free). " 48.88, " 8.7, " " 9.92



## PLASMA PROTEIN GIVEN BY VEIN AND ITS INFLUENCE UPON BODY METABOLISM\*

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Experiments in this laboratory (5, 9) gave evidence that under certain conditions the *plasma proteins* as plasma given by vein are utilized by the fasting dog to replace or repair *body protein*. Experiments tabulated below confirm and extend this thesis. The body mechanism may be seriously disturbed and there may develop a peculiar intoxication (somewhat like that noted after long periods of fasting) when large amounts of plasma are given by vein and only small amounts of sugar by mouth (Tables II and II-A). There is obvious increase of protein catabolism and the urinary nitrogen may rise to very high levels (Table II).

In considering the fate of the injected plasma protein we may mention two possibilities: that it is stored as plasma protein or as intermediates; that it is modified and incorporated in tissue cells. It seems probable that both mechanisms are involved.

### *Methods*

The dogs used in these experiments were active, healthy, and under observation in the experimental colony for years. During the experiments the dogs were kept in galvanized iron metabolism cages in the laboratory under constant supervision. A 2 or 3 day fast in each experiment preceded the experimental observations. Water was present in the cages at all times. Food intake is shown in the clinical histories.

Dog plasma for injection was obtained by blood collection into heparin from healthy donors. This plasma was injected into the jugular vein in one or two doses each day. Plasma protein,

\* Aided by a fund from Eli Lilly and Company.



as given in Tables I to III, was figured on the basis of the non-protein nitrogen as 20 mg. per cent.

All periods, as given in Tables I to III, were terminated by catheterization and rinsing of the bladder. The urine was collected several times each day as passed in the cage and preserved by toluene and refrigeration. Urine analyses were made daily or every 2nd day: total nitrogen by macro-Kjeldahl, ammonia by acration, urea by urease, creatine and creatinine by the methods of Folin, and uric acid by the method of Morris and Macleod (2).

The Cowgill diet (8) as used contained sucrose 55.1 gm., dextrin 18.17, lard 16.65, butter 6.54, bone ash 2.36, a salt mixture (10), and supplements to supply vitamins A, D, B, G. For vitamins A and D two tablets of cod liver oil concentrate (Health Products Corporation, Newark) were given daily per 5 to 7 kilos of body weight of the dog. For vitamins B and G the following solution was administered: an aqueous suspension of 75 gm. of liver extract No. 343 (Eli Lilly and Company) and 20 gm. of ryzamin in 250 cc. of water. This suspension, adjusted to a pH just acid to litmus, was given orally in the dosage of 0.7 cc. per kilo of body weight. This mixture contains 132 mg. of nitrogen per 100 gm. of the diet mixture and only very small amounts of "protein," 4.93 calories per gm. Other details of the method relating to plasma volume, hemoglobin, and animal care have been recently described (11).

### *Experimental Observations*

Tables I and I-A give all the experimental data relating to a long experiment with intravenous injections of large amounts of normal dog plasma. About 14 gm. of protein or 2.3 gm. of nitrogen are given daily without clinical disturbance and the dog during this time is in nitrogenous equilibrium (34 gm. of nitrogen excreted in the urine and 37 gm. of nitrogen in the protein injected during this 18 day period (Table I-A)). The weight loss amounts to 0.3 kilo. There is the usual shrinkage of the plasma volume noted during fasting or associated with very low protein diets. The non-protein nitrogen remains at a normal level. The fall in the hemoglobin level is due to repeated samplings and the determination of blood volume. The rise in circulating plasma

protein from 7 per cent to 8.6 per cent is the usual reaction and this level falls to normal a few days after discontinuance of the plasma injections.

Table I shows the slight excess in urinary nitrogen which appears as the plasma injections are begun. This is probably less than the increase which would appear if the same amounts of plasma protein were fed by mouth. When the plasma injections are discontinued, the urinary nitrogen promptly falls to the expected fasting level—in other words there is no escape of nitrogen in the after period, as would follow if the plasma protein was catabolized and eliminated in the urine as is true for foreign protein (9).

Table I also shows a drop in the urea-ammonia fraction of the total nitrogen from 70 to 80 per cent noted in the fasting dog (2) to .65 per cent noted at times during plasma injections. This type of reaction was designated as the "reaction of conservation" and has been reported in other experiments (2) which showed a similar reaction when the anemic dog built much new hemoglobin during fasting periods.

It is to be recalled that this dog in the week preceding this experiment had received a diet of liver, liver extract, and iron to promote a very lively hemoglobin production and a return to a high normal level of hemoglobin. It has been shown that under such circumstances the dog will put away a considerable *reserve store* in its body from which it can build much hemoglobin. Also on this diet the dog will store away protein materials from which much new plasma protein can be fabricated during periods of low plasma protein due to plasmapheresis. Under such circumstances with the *reserve stores* well filled, it is probable that the body would accept with less ease large surplus amounts of plasma protein; yet this dog can do so readily, as shown in Table I-A.

Tables II and II-A at first sight appear to be confused by a number of abnormal factors, but we believe when subjected to analysis several important conclusions emerge. This dog had been used as a blood donor and although the hemoglobin level was normal the dog was below normal weight. There was no frank clinical abnormality. During the *fore period* of 2 days fast and 6 days of sugar (50 gm. per day) feeding the weight loss was rapid and the urinary nitrogen was somewhat above the expected levels.

TABLE I

*Nitrogen Equilibrium with Plasma Protein by Vein*

Dog 32-3, adult, female mongrel coach dog.

Period No. (48 hrs.)	Total N	Urea N + NH <sub>2</sub> -N		Creatinine N	Creatine N	Uric acid N	Undetermined N	
	gm.	gm.	per cent	mg.	mg.	mg.	mg.	per cent
1*	7.65	6.42	83.9	333	100	23	770	10.2
2	4.69	3.63	77.4	321	74	21	640	13.8
3	3.59	2.56	71.3	300	71	21	640	17.7

## Plasma injections begun plus Cowgill diet

4	4.06	3.07	75.6	307	81	21	580	14.3
5	3.01	2.03	67.4	294	26	18	640	21.3
6	3.24	2.24	69.1	287	27	19	670	20.6
7	3.20	2.22	69.4	276	20	19	670	20.8
8	3.58	2.48	69.3	284	7	20	790	22.0
9	4.50	3.16	70.2	282	8	20	1030	22.9
10	3.86	2.50	64.8	271	11	19	1060	27.4
11	4.19	2.88	68.7	265	26	22	1000	23.9
12	4.50	2.94	65.3	273	21	22	1240	27.6

## Plasma injections discontinued

13	3.17	2.03	64.0	243	12	18	870	27.4
14	2.79	1.82	65.2	255	26	19	670	24.0
15	2.90	2.10	72.4	250	19	21	510	17.6

Figures in columns headed gm. or mg. represent nitrogen excreted in successive 48 hour periods.

\* Period 1 = 2nd and 3rd days of fasting.

*Clinical Experimental History (Tables I and I-A)*

Dog 32-3, adult female mongrel bull-coach. Born April, 1932. Raised on beef muscle diet from weaning until 1 year of age. Experimental anemia due to blood loss April, 1933, to March, 1934. Permitted to return to normal hemoglobin value for metabolism experiment. Rest period on kennel diet to September, 1936. September 30, 1936, preparation for plasma protein depletion. Vitavose, fat, and low protein diet. Plasmapheresis November 16, 1936, to January 12, 1937. Again rendered anemic and hemoglobin reserve removed. Standard salmon-bread ration. Routine anemia experiments to May 11, 1937.

May 11. Blood picture permitted to go back to normal in preparation for plasma injection experiment. Daily diet consisted of pig liver 300

TABLE I-A

*Plasma Protein by Vein Utilized in Body Metabolism*

Dog 32-3, adult, female mongrel coach dog.

Period No. (48 hrs.)	Urinary total N	Plasma injected		Circulating plasma			Hemo- globin	Weight
		Total N	Protein	Protein	Non-pro- tein N	Total volume		
	gm.	gm.	gm.	gm. per cent	mg. per cent	cc.	per cent	kg.
1*	7.65					728	107	14.2
2	4.69							
3	3.59							

## Plasma injections begun

4	4.06	4.93	30.3	7.0	17	616	104	13.7
5	3.01	4.59	28.1	7.7				
6	3.24	4.61	28.2	8.3				13.5
7	3.20	2.36	14.5	8.6				
8	3.58	4.70	28.8	8.6	19	625	96	13.6
9	4.50	4.65	28.5	8.9				
10	3.86	2.18	13.3	8.7				
11	4.19	4.51	27.6	8.3		570	90	13.4
12	4.50	4.81	29.5	8.6	18			

## Plasma injections discontinued

13	3.17							
14	2.79							
15	2.90							13.1
16						617	78	12.9

\* Period 1 = 2nd and 3rd days of fasting.

gm., Iextron 6 gm. (liver-stomach concentrate 4.3 gm., vitamin B complex 0.16 gm., including 330 mg. of iron), and salmon-bread.

May 17. Fasting begun.

May 18. Blood volume showed hemoglobin still below normal; transfusion of normal blood (30 gm. of hemoglobin).

*Metabolism Experiment*—3 days fast, followed by 4 days feeding of Cowgill diet (8) 200 gm. daily but without vitamin supplement. 18 day period of intravenous injection of heparinized dog plasma, 225 cc. daily except Sundays. Cowgill diet without vitamin supplement during injection period and for 6 day after period. For weight loss see Table I-A. Dog was in good condition at the end of the experiment.

TABLE II

*Plasma Injections Cause Intoxication. Controlled by Diet*

Dog 34-146, adult, female white bull dog.

Period No. (48 hrs.)	Total N	Urea N + NH <sub>4</sub> -N		Creati- nine N	Creatine N	Uric acid N	Undetermined N	
	gm.	gm.	per cent	mg.	mg.	mg.	mg.	per cent
1*	8.73	7.80	89.4	387	129	15	400	4.5
2	7.58	6.97	92.0	331	96	12	170	2.1
3	6.37	5.67	89.0	291	26	11	370	5.8
4	6.04	5.39	89.2	275	26	12	340	5.6

Plasma injections begun; sugar by mouth

5	11.11	9.82	88.4	364	221	28	680	6.0
6	10.39	9.35	90.0	305	146	23	570	5.5
7	14.29	12.95	90.7	319	208	26	790	5.4
8	17.49	15.55	88.9	295	340	36	1270	7.3

Plasma injections continued plus Cowgill diet

9	9.62	8.52	88.6	276	219	24	580	5.9
10	2.89	1.99	68.8	248	28	14	610	21.2
11	3.70	2.70	73.0	285	26	17	670	18.1
12	3.47	2.49	71.9	277	13	12	680	19.4
13	2.61	1.68	64.5	261	16	10	640	24.5
14	2.22	1.22	55.1	261	24	8	710	31.7
15	2.01	1.14	56.8	239	14	7	610	30.3
16	2.34	1.36	58.2	255	21	7	700	29.7
17	2.34	1.47	62.7	250	19	7	590	25.5
18	2.36	1.49	63.3	238	10	7	620	25.9

Plasma injections discontinued

19	2.44	1.49	61.0	255	31	—	660	26.9
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\* Period 1 = 2nd day of fasting (24 hours). The figures expressed in gm. or mg. for this period have been doubled.

*Clinical Experimental History (Tables II and II-A)*

Dog 34-146, adult female white bull. Born December, 1933. Used as blood donor. Plasma injections begun March 1, 1937. Kennel diet until beginning of experiment. 2 days fasting. 6 days 50 gm. of dextrose by stomach tube daily in the fore period. 28 day period of intravenous injection of heparinized dog plasma, 1081 cc. during the 1st week and 1200 cc. during the 2nd week, average daily injection 163 cc. First 8 days of plasma

TABLE II-A

*Positive Nitrogen Balance Due to Plasma Protein by Vein*  
Dog 34-146, adult, female white bull dog.

Period No. (48 hrs.)	Urinary total N	Plasma injected		Circulating plasma			Hemo- globin	Weight
		Total N	Protein	Protein	Non- protein N	Total volume		
	gm.	gm.	gm.	gm. per cent	mg. per cent	cc.	per cent	kg.
1	8.73			7.1	24	695		15.9
2	7.58							
3	6.37							
4	6.04					602	149	14.5

Plasma injections begun; sugar by mouth

5	11.11	3.66	22.5	7.0	27			14.4
6	10.39	3.59	22.1	7.7				
7	14.29	3.44	21.0	7.9				
8	17.49	3.37	20.7	8.3	35	598	132	13.0

Plasma injections plus Cowgill diet

9	9.62	4.36	26.7	8.5				
10	2.89	4.11	25.2	7.7				
11	3.70	2.21	13.6	7.2				
12	3.47	4.12	25.3	7.5		665	109	13.3
13	2.61	4.42	27.1	7.9	18			
14	2.22	4.17	25.6	8.0				
15	2.01	2.14	13.1	7.6		639	106	13.2
16	2.34	4.24	26.0	8.2	17			
17	2.34	4.12	25.2	8.4				
18	2.36	2.02	12.4	8.5				

Plasma injections discontinued; Cowgill diet continued

19	2.44					687	98	13.0
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injection period 50 gm. of dextrose by stomach tube daily. Remaining 20 days of injection period and 2 days after period dog was given Cowgill diet, 200 gm. daily. 25 cc. of packed red cells added to plasma for injection on 2 days because of drop in red cell hematocrit and hemoglobin. Dog thin at beginning of experiment and lost weight rapidly until the Cowgill diet was fed. Thereafter weight was maintained. Dog thin but in good condition at end of experiment.

Plasma injections (about 160 cc. per day) and sugar by mouth during Periods 5 to 8 at once gave evidence of abnormality. Rapid weight loss continued and the urinary nitrogen rose day by day from 11.1 to 17.5 gm. The urea-ammonia fraction remained at a very high level (90 per cent) and there was a sharp increase in the creatine output which we believe is an indication of injury of muscle tissue (3). The total nitrogen rises to such high levels that it cannot be explained by simple waste elimination of the injected plasma protein but indicates clearly that toxic products are formed in this reaction which injure body protein and swell the total output of protein split-products. The non-protein nitrogen rose during this period of intoxication and promptly fell when the Cowgill diet replaced the sugar feeding.

A liberal intake of fat and carbohydrate (Cowgill diet) changes the picture completely, although the plasma injections are continued as before. The dog was clinically improved and following Period 9, which is a carry-over from the period of intoxication, we note very low levels for urinary nitrogen and a conspicuous positive nitrogen balance. During Periods 10 to 18 inclusive (18 days) the nitrogen intake as plasma protein is 31.5 gm. and the urinary nitrogen is 23.9 gm. Small amounts of nitrogen in the Cowgill diet and feces do not change this picture of active nitrogen conservation. There is a slight gain in weight during this period (Table II-A). The creatine falls promptly to very low levels (Table II) and the urea-ammonia percentage falls rapidly to 55 to 60 per cent.

Dog 34-146 (Tables II and II-A) conserves nitrogen more conspicuously than the other two dogs. As an explanation we point to Periods 5 to 8 when there was definite intoxication and a strong negative nitrogen balance. It is probable that there was a depletion of body stores of protein during this period and the body therefore was ready to use protein materials to replete these stores and repair the protein injury. Obviously the presence of adequate fat and carbohydrate is a very important factor in this reaction of conservation.

Dog 32-131 had been used in 1932 (4) and again in 1934 (9) for experiments of a similar type. Plasma had been given by vein during 2 week periods with definite evidence of plasma protein utilization but with no evidence of intoxication. There was ap-

proximately a nitrogenous equilibrium during the plasma injection periods and no evidence of subsequent wastage of nitrogen.

TABLE III

*Intoxication Due to Plasma Protein Injections by Vein*

Dog 32-131, adult, mongrel hound.

Period No. (48 hrs.*)	Total N	Urea N + NH <sub>3</sub> -N		Creatinine N	Creatinine N	Uric acid N	Undetermined N	Plasma injected		Weight
		gm.	per cent					Nitrogen	Protein	
	gm.	gm.	per cent	mg.	mg.	mg.	per cent	gm.	gm.	kg.
1	7.43	5.81	78.2	430	171	46	13.1			22.1
2	6.42	5.05	78.7	385	170	39	12.0			
3	5.37	4.16	77.5	364	63	38	13.8			21.0
4	4.70	3.59	76.3	364	59	34	14.0			21.1
5	4.31	3.15	73.2	379	35	36	16.4			20.7
Plasma injections begun; sugar by mouth										
6	5.37	4.15	77.3	387	124	48	12.3	5.81	35.6	20.5
7	5.07	3.81	75.2	361	63	40	15.7	4.82	29.5	20.3
8	5.60	4.46	79.6	344	20	40	13.2	5.34	32.7	20.1
9	5.75	4.43	77.2	354	18	42	15.6	5.25	32.2	19.8
10	7.01	5.74	81.9	344	20	44	12.3	5.09	31.1	
11	8.76	6.63	75.7	354	33	51	19.3	4.90	30.9	19.4
12	12.30	7.98	64.9	350	41	52	31.6	6.23	38.3	
Plasma injections discontinued; plasma plus sugar by mouth										
13	9.31	7.02	75.4	323	31	40	20.4			18.4
14	7.10	5.82	82.0	320	36	38	12.5			
15	6.58	5.59	85.0	317	44	37	8.9			
Plasma by mouth discontinued; sugar only by mouth										
16	6.30	5.32	84.5	314	25	36	9.5			17.8
17	4.97	3.83	77.1	321	16	31	15.5			17.7

\* Each period 48 hours except Nos. 1, 5, and 15 which are each 24 hours but corrected for 48 hours. Period 1 = 1st day of fasting and Period 2 = 2nd and 3rd days of fasting.

*Clinical Experimental History (Table III)*

Dog 32-131, normal adult mongrel hound used for various plasmapheresis experiments (4, 9). Dog was given regular kennel ration (kitchen scraps) until beginning of experiment; then 3 days of fasting were followed by 5 days feeding of 50 gm. of dextrose by stomach tube daily. Plasma injec-



tions intravenously 14 days (240 to 300 cc., average 257 cc. of heparinized dog plasma). Plasma usually given in one dose, sometimes divided into two doses dependent upon amount injected and clinical reaction of animal. Sugar feeding was continued during plasma injection period; followed by 5 days plasma feeding plus dextrose (50 gm. of dextrose plus 250 cc. of dog plasma daily by stomach tube). Then 4 days sugar feeding alone (50 gm. of dextrose as before). Dog vomited 10 out of 14 days following plasma injections. Urine contaminated with vomitus in Period 8 only. Dog in good condition at end of experiment.

The same dog under slightly different conditions (Table III) does show evidence of mild intoxication during the last three periods of plasma injection (Periods 10 to 12) and there is a conspicuous rise in the total urinary nitrogen and undetermined nitrogen. It should be noted that in this last experiment (Table III) the dog received more protein (230 gm.) than in the earlier experiments (9) when 160 gm. were given during the same 14 day period. It is possible that the body can handle only about so much plasma protein and beyond this point the injected protein is catabolized with a related mild intoxication. It is also probable that increased ingestion of fat and carbohydrate will enable the dog to utilize more plasma protein given as plasma by vein (Tables II and II-A).

Feeding of plasma by mouth gives a rise in urinary nitrogen but a part of the high nitrogen output in Periods 13 and 14 is a carry-over from the intoxication in the three preceding periods. Even in the last period the urinary nitrogen has not fallen to the expected low fasting level. During this experiment there was a considerable weight loss (4.4 kilos) which supports the argument for intoxication.

In a discussion of this experiment (Table III) some observers would perhaps argue that simple storage of plasma protein was the predominant factor, but we believe that intoxication was largely responsible for the reaction given in Table III.

#### DISCUSSION

We propose for consideration the following thesis to explain the observed facts as tabulated above relating to the intravenous injection of blood plasma and its subsequent fate in the body. For plasma protein to be stored or utilized in the liver, muscles, or body tissues, it must be stored as such or slightly catabolized (large aggregates rather than amino acids) and synthesized to

precipitate is centrifuged and washed three times in the centrifuge tube with 100 ml. of water, and is then suspended in acetone and filtered, suction being continued until a moist coherent cake is secured. It should not be allowed to dry. The product is transferred to a small beaker and thoroughly triturated into a thin paste with 20 ml. of anhydrous methyl alcohol that contains 4 per cent of hydrogen chloride. The paste is transferred with the aid of a little methyl alcohol to a centrifuge tube and centrifuged. The precipitate of lead chloride is disintegrated and washed twice with 20 ml. of acid methyl alcohol and once with 10 ml. of anhydrous methyl alcohol. The clear combined extracts are then treated with 5 to 6 volumes of ether previously *freed from peroxide* (3) and the mixture is chilled for several hours. The precipitate is filtered off, washed with purified ether, and dried over sulfuric acid in a vacuum desiccator; yield, 0.5 to 0.7 gm.

*Crystallization*—The preceding steps can be carried out successfully on a much larger scale than is described without grave losses of pigment, but the final crystallization from water is wasteful. 500 mg. of the purified pigment are suspended in 4.5 ml. of water and triturated with a thermometer until the particles are completely disintegrated. The beaker is then heated in a water bath at 80° until the temperature of its contents reaches 70°; 0.5 ml. of 0.1 N hydrochloric acid is added, and stirring is continued until the temperature reaches 75–76°. The solution is rapidly filtered through a thin pad of asbestos in a small hot water funnel, and the residue is washed with 1 to 2 ml. of hot water. The clear filtrate is chilled for 6 hours, and the product is filtered off and washed successively with a little ice water, with absolute alcohol, and with purified ether. It is then dried *in vacuo* over sulfuric acid overnight, pulverized in an agate mortar, and dried to constant weight over phosphorus pentoxide at *room temperature*; yield, 0.2 to 0.3 gm.

Another excellent method to purify the material further is to repeat the precipitation from methyl alcohol solution with ether. The solution must be centrifuged perfectly clear; the recovery in this case is about 75 per cent.

*Discussion of Procedure*—Ethyl alcohol is for several reasons more satisfactory than methyl alcohol in the early stages of the preparation, but methyl alcohol is to be preferred in the later

stages. The relative quantities of acid recommended must be adhered to in order that the dilution from the addition of aqueous lithium hydroxide shall not be excessive; otherwise serious losses may be encountered. Lithium hydroxide is employed because of the solubility of lithium chloride in alcohol; the concentrate contains little or no inorganic halide when prepared as described, but is grossly contaminated if sodium hydroxide is used. The lead acetate precipitate becomes colloidal if washed with alcohol, and excessive washing with acetone must be avoided for the same reason. The decomposition of the lead precipitate with acid methyl alcohol is effective only if the solid is very carefully triturated. The use of ether freed from peroxide is essential if oxidation and partial destruction of the pigment are to be avoided. The product derived from the decomposition of the lead precipitate contains from 5 to 10 per cent of impurities which are mainly inorganic, and the final separation from hot water is necessary only if preparations for analysis are required.

The order of magnitude of the losses involved in the procedure may be appreciated from the following average figures. 1 kilo of fresh beet-root yielded 155 gm. of dry tissue and 119 gm. of alcohol-extracted dry residuc. Acid ethyl alcohol dissolved 25.4 gm. of this, and 8.24 gm. of pigment concentrate were secured on neutralization. This contained 1.31 gm. of pigment, as calculated from the extinction coefficient, and 28 per cent of ash, much of which consisted of water-soluble phosphates. After lead acetate purification, the preparation weighed 1.08 gm. and contained about 5 per cent of ash. The final purification from water gave 0.5 gm. or about 40 per cent of the pigment in the crude concentrate. The yield from sixteen preparations was 6 to 6.8 gm. of pigment purified by the lead acetate procedure per kilo of dry tissue, and 3 to 4 gm. after final purification. Schudel reported a yield of 1.3 gm. from 1 kilo of dry beet-root, and Ainley and Robinson 0.3 gm. from 56 kilos of fresh root (approximately 4 kilos of dry tissue). Their methods of preparation are, however, entirely different from ours.

*Properties of Pigment*—The finely ground purified product is nearly black with a pronounced green luster and closely resembles such a dye as crystal violet in appearance. The streak is purplish red. Under the microscope, the masses show many light-reflect-

cell protein. This mechanism may be disturbed by overloading (injection of too much plasma) or lack of adequate carbohydrate and fat. As a result the catabolism might go too far within or without the body cells and produce toxic split-products with evidence of intoxication and a great surplus of urinary nitrogen even much above that of the introduced protein. The experimental data in the accompanying paper (Howland and Hawkins (6)) are in harmony with this thesis.

It is generally accepted that there are protein stores within the body which can be drawn upon in emergencies, for example fasting, acute anemia, and early plasma depletion (plasma-pheresis). Some of this store may be in body fluids including plasma, but a large amount must be in cells of the body (1, 7) (liver, muscle, and other tissues). We do not visualize these stores as coal in storage bins but as protein material related to the cell protein matrix—perhaps of slightly different character or as large aggregates approximating proteins in size. We visualize no difficulty in an intracellular modification and utilization of storage protein under favorable conditions to repair or replace the cell protein matrix.

Even if one argues that the plasma protein is stored as such, it must be stored within the cells of the organs and tissues where it could scarcely escape modification by the cells concerned. The fact that the blood plasma protein concentration falls to normal in 2 to 4 days after injections are discontinued would speak emphatically for normal protein concentration within the body extracellular fluid at that time and exclude this body fluid as a hypothetical protein storage reservoir.

#### SUMMARY

Dog plasma given by vein to a protein-fasting dog is well utilized under certain conditions and will maintain the dog in nitrogenous equilibrium. There is no wastage of surplus urinary nitrogen in after periods. It appears that the introduced plasma protein under such conditions is utilized efficiently in body metabolism to replace or repair organ or tissue protein. One can scarcely escape the conclusion that this mechanism is operative under normal as well as emergency conditions. This has suggested a "dynamic equilibrium" between plasma proteins and cell proteins.

This mechanism may be disturbed, and associated with plasma

injection there may appear an intoxication. It is possible that overloading by large plasma injections is one factor which may bring about intoxication, probably related to catabolism of the excess injected plasma protein. Administration of abundant carbohydrate and fat will prevent the intoxication.

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## PROTEIN METABOLISM, PROTEIN INTERCHANGE, AND UTILIZATION IN PHLORHIZINIZED DOGS

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Can the body utilize plasma proteins in its metabolic processes when there is a demand for protein material? Previous reports from this laboratory have shown that dog plasma given intravenously to a protein-fasting dog will maintain the dog approximately in nitrogen equilibrium. No surplus nitrogen elimination is found in subsequent periods. Such evidence points to an efficient utilization of the introduced plasma protein either to replace or repair tissue protein (2, 9, 3).

Further knowledge as to the fate of this injected plasma protein is desirable. Is it metabolized in the same manner as it would be if it were fed to the dog? It seemed possible that additional evidence as to the mode of utilization might be obtained by introducing the plasma protein into a dog rendered diabetic by phlorhizin. If it should be catabolized in the usual manner, then there should be an increased amount of nitrogen and sugar eliminated in the urine. Lusk (6) has shown that dogs rendered completely diabetic by phlorhizin convert 58 per cent of fed proteins to glucose and Janney (4) has demonstrated that 55 per cent of fed serum protein is converted into glucose.

The metabolism of injected plasma protein apparently is different. It promptly disappears from the blood stream and yet no excess nitrogen or sugar is recovered from the urine. Either the protein is removed from the blood stream and stored in body tissues in its original form or else it is only partially broken down and then rebuilt into tissue proteins. It is obvious that it is not catabolized to amino acids before being utilized, since no excess nitrogen or sugar is found.

This suggests an interesting method of protein exchange within

the body—not a complete protein catabolism as in digestion but a slight catabolic change of serum protein into large aggregates of amino acids with subsequent reassembly into the protein peculiar to the cell of the particular organ or tissue.

### *Methods*

The plan of the individual experiment was simple. The dog was fasted for 24 hours and then with continued fasting, phlorhizin in olive oil in 1 gm. doses was injected subcutaneously daily throughout the experimental period. On the 3rd or 4th day after phlorhizin injection was begun, when the D:N ratio indicated the dog had been rendered completely diabetic, urine collected over a 12 hour basal period was taken and determinations carried out. Following the basal period a known amount of plasma protein was injected intravenously and the animal followed through an adequate number of periods all 12 hours in length. Control experiments were conducted under identical conditions, a similar amount of Locke's solution (300 cc.) containing a known amount of anhydrous glucose (300 mg.) being used. The dogs were kept in metabolism cages with access to water. At the end of each period the dog was catheterized and all urine removed with washing out of the bladder with water until the return fluid was found to be water-clear. Toluene (5 cc.) was used as preservative.

Total blood plasma nitrogen, albumin and globulin, non-protein nitrogen, sugar, and acetone bodies were determined. Total urinary nitrogen, urea, ammonia, acetone bodies, and sugar were determined.

Blood for analysis was drawn from the jugular vein into hematocrit tubes containing a 1.4 per cent solution of sodium oxalate and centrifuged for 35 minutes. Total nitrogen of the plasma obtained was determined by the macro-Kjeldahl method,  $K_2SO_4$  and selenious acid being used as the oxidizing agent. 1 cc. of plasma was used and determinations were run in triplicate. The determination of albumin and globulin was carried out according to Howe's method, as described by Peters and Van Slyke, with 22 per cent sodium sulfate at 37°; triplicate determinations were carried out.

Urine was analyzed for total nitrogen on 1 cc. aliquots by the macro-Kjeldahl method.

The urease method with aeration and titration for both urea and ammonia, according to Van Slyke and Cullen, was utilized in determining urine urea and ammonia.

Blood filtrate was obtained by the Folin and Wu tungstic acid precipitation method as modified by Van Slyke and Hawkins. Non-protein nitrogen was determined by nesslerization.

The colorimetric copper method of Benedict was used for blood sugar and Shaffer and Hartmann's copper titration method for urine sugar.

Titrimetric determination of the acetone bodies in blood and urine with Denigès' reagent were made as described by Van Slyke and Fitz. The Folin-Wu blood precipitation method was used in place of the mercuric sulfate after careful check determinations.

Blood volumes were determined by the brilliant vital red dye method.

Plasma for injection was obtained from donor dogs, heparin being used as anticoagulant, with centrifugation of blood for 35 minutes. Total plasma nitrogen and sugar were determined. The plasma was given intravenously from a gravity bottle.

The phlorhizin utilized was a preparation of either Merck or Schering-Kahlbaum. It was first recrystallized from an alcohol-water solution, filtered, and dried over  $H_2SO_4$  in a vacuum desiccator. With sterile technique this was ground in a mortar with olive oil which had previously been immersed in a boiling water bath for 30 minutes. 1 gm. of phlorhizin mixed in 7 cc. of olive oil was the daily dose injected at 24 hour intervals.

The animals used in the experiments were mongrel dogs of approximately 15 kilos weight. Between experiments these dogs were on a kennel diet of hospital scraps for at least 6 weeks, during which time their former weight was completely regained.

### *Experimental Observations*

Table I (Dog 35-13) illustrates the results obtained from both the feeding and injection of plasma protein. As soon as the dog had been made diabetic by phlorhizin, a 12 hour basal period collection was made and analysis performed. At the beginning of the next collection period the dog was given 302 cc. of dog plasma by stomach tube. This plasma contained 3.36 gm. of nitrogen, 20.84 gm. of protein, and 0.25 gm. of sugar. At the end of this period 1.97 gm. of extra nitrogen and 9.48 gm. of extra



sugar were present in the urine above the control basal level. The extra nitrogen amounts to 58 per cent of the fed nitrogen. On the basis of this extra nitrogen one would expect an increase of 7.2 gm. of sugar, whereas 9.48 gm. were recovered. Accompanying the presence of the excess sugar the acetone bodies of both blood and urine drop markedly. These data show that plasma protein when fed is digested and partially converted into sugar just as other proteins are. Since the D:N ratio had re-

TABLE I  
*Plasma by Stomach and Vein*

Dog 35-13.

Period No.	Urine			Acetone bodies		Plasma protein	Plasma volume	D:N ratio
	Total N	Urea N + NH <sub>3</sub> -N	Sugar	Blood	Urine			
	gm.	per cent	gm.	mg. per cent	gm.	gm. per cent	cc.	
1						6.14	705	
2	5.82	60	22.03	103	2.95	5.27		3.78
Plasma by stomach tube 302 cc. = 3.36 gm. N = 20.84 gm. protein								
3	7.79	83	31.76	50	0.90	4.99		4.08
4	5.13	77	18.47	69	1.25	5.39	641	3.60
Plasma by vein 278 cc. = 3.08 gm. N = 19.0 gm. protein								
5	4.96	76	19.77	49	0.86	5.57		3.98
6	4.47	84	19.01	58	0.72	6.01		4.26
7	3.31	76	17.18	67	0.40	5.82		5.19
8	3.73	87	15.15	46	0.26	6.12	638	4.06

turned to 3.6 at the end of the subsequent basal period, 278 cc. of dog plasma were injected into the jugular vein at the beginning of the next period.

The plasma was given in two doses with an hour elapsing between the first and second injections. It contained 3.08 gm. of nitrogen, 19.00 gm. of protein, and 0.26 gm. of sugar. In the urine collected at the end of this period there was no excess nitrogen or sugar. In subsequent basal periods the total nitrogen excreted decreased and the sugar diminished slightly. Blood and

urine acetone bodies are low as compared with the original basal period. Owing to lack of space all data cannot be shown in Table I. The weight at the beginning was 14.7 kilos and there was a gradual daily loss to 13.4 kilos. The red cell hematocrit also decreased from 48.9 to 39.8 per cent. Approximately 25 cc. of blood were removed daily for analysis. The albumin to globulin ratio determined daily showed no significant alterations. The blood sugar varied from 24 to 30 mg. per cent. .

As the experiment progressed the dog became lethargic, but shortly after the injection of the plasma it became more alert and active. At the site of injection of one of the doses of phlorhizin a small abscess developed on the next to the last day of the experiment. It was lanced and 15 cc. of reddish semifluid material were obtained. On the morning of the last day, while being catheterized, the dog had some convulsions with loss of sphincter control. This is no doubt related to the hypoglycemia. The dog promptly returned to normal upon feeding.

This experiment illustrates two phenomena. By calculation the total circulating plasma proteins were determined before and after injection. By addition of the amount injected (19 gm.) and deduction of the amount removed in sampling (3.7 gm.) it is found that a total of 10.78 gm. of the injected protein has disappeared from the blood stream without any evidence of its having been catabolized. Also following the injection of the protein the total urine nitrogen decreases in each basal period. This suggests conservation of nitrogen on the part of the dog.

After 2 months rest a repeat experiment (Table II) was performed on this dog, except that feeding of plasma protein was excluded. After three 12 hour basal periods 340 cc. of plasma were injected intravenously in two divided doses early during Period 4. This plasma contained 3.88 gm. of nitrogen, 24 gm. of protein, and 0.31 gm. of sugar. Following the injection the dog became more active and alert. At the end of this 12 hour period the plasma protein percentage was elevated from the control level of 5.94 gm. to 6.55 gm. The urine nitrogen shows a distinct decrease as compared with the control levels and there is a slight decrease in the urine sugar. At the end of the subsequent basal period there is a very marked decrease in both urine nitrogen and sugar. This marked reduction in the amount of nitrogen and

sugar seemed unlikely and loss of some urine was considered. The volume of urine, however, was approximately the same as during other collection periods and there was no evidence of urine loss in the region of the cage. Calculations were checked and found to be correct, so we have to accept the figures as given although we do so with some reservations. During the last two basal periods the urine sugar and nitrogen rise but are still much below the control basal levels. The acetone bodies of the blood decreased slightly, while those in the urine are much lower than the control levels. There was gradual progressive weight loss

TABLE II  
*Plasma by Vein*

Dog 35-13.

Period No.	Urine			Acetone bodies		Plasma protein	Plasma volume	D:N ratio
	Total N	Urea N + NH <sub>3</sub> -N	Sugar	Blood	Urine			
	gm.	per cent	gm.	mg. per cent	gm.	gm. per cent	cc.	
1	4.71	78	18.30	75	2.95	5.79		3.88
2	5.17	78	17.88	75	3.46	6.15		3.46
3	5.09	78	17.64	68	2.58	5.94	694	3.46
Plasma by vein 340 cc. = 3.88 gm. N = 24 gm. protein								
4	3.74	76	15.74	47	0.92	6.55		4.21
5	1.22	80	4.54	18	0.47	6.42	641	3.70
6	2.92	83	12.28	60	0.36	6.29		4.21
7	3.44	84	13.05	67	0.47	6.21		3.79

from 15.2 to 14.1 kilos. Blood sugar levels ranged between 19 and 22 mg. per cent. Non-protein N is present in normal amount.

In this experiment the entire amount of injected plasma protein disappeared from the blood stream within 24 hours, and there is indication of nitrogen conservation.

Dog 35-15 (Table III) was phlorhizinized in a similar manner and then given 355 cc. of plasma which contained 4.01 gm. of nitrogen, 24.74 gm. of protein, and 0.316 gm. of sugar. Non-protein N = 0.017 gm. At the end of the period in which the injection was made the plasma protein circulating was but slightly elevated. In the urine 0.414 gm. of nitrogen in excess was ob-

tained but only 1.14 gm. of sugar, part of which can be accounted for by the 0.316 gm. given in the plasma. In subsequent periods the amount of nitrogen and sugar eliminated is definitely below the control values. 48 hours after the plasma was injected the total circulating protein is 37.29 gm. which is 5.74 gm. less than that circulating just previous to injection. 3.92 gm. of protein were removed in the samples taken. Again there has been complete disappearance of all of the injected protein with no excess of nitrogen or sugar in the urine. Blood and urine acetone bodies were decreased after injection but rose in subsequent periods.

TABLE III  
*Plasma by Vein*

Dog 35-15.

Period No.	Urine			Acetone bodies		Plasma protein	Plasma volume	D:N ratio
	Total N	Urea N + NH <sub>3</sub> -N	Sugar	Blood	Urine			
	gm.	per cent	gm.	mg. per cent	gm.	gm. per cent	cc.	
1						6.08	643	
2	4.59	76	17.85	89	1.50	6.40	673	3.88
Plasma by vein 355 cc. = 4.01 gm. N = 24.74 gm. protein								
3	5.01	77	18.99	56	0.99	6.55		3.79
4	3.11	78	13.75	91	0.49	6.99		4.41
5	3.05	73	11.21	105	1.01			3.67
6	3.22	76	12.38	99	1.28	6.64	562	3.84

Non-protein N was not elevated following injection. The weight dropped from 14.95 to 14.15 kilos and the red cell hematocrit from 46.2 to 43 per cent. The albumin to globulin ratio showed no significant change. The blood sugar ranged from 22 to 32 mg. per cent.

Table IV (Dog 35-127) gives results on another dog that weighed 10.5 kilos as compared with about 14.5 kilos for the other dogs. Only 260 cc. of plasma were injected and it contained 3.18 gm. of nitrogen, 19.08 gm. of protein, and 0.23 gm. of sugar. The plasma was injected in one dose over a 15 minute period with no untoward effects, but an hour later the dog vomited and def-

ecated and appeared inactive. Recovery was prompt and there was no other disturbance. Following the injection there is no excess nitrogen or sugar in the urine and in subsequent basal periods the nitrogen and sugar levels gradually decrease in amount. There is no striking change in the blood and urine acetone bodies. Non-protein N remained at normal levels and the blood sugar ranged from 10 to 32 mg. per cent. The weight fell gradually from 10.7 to 9.64 kilos.

Within 48 hours of the injection practically all the injected plasma protein has disappeared from the blood stream. 3.09 gm. of protein were removed in samples taken for analysis. Again

TABLE IV  
*Plasma by Vein*

Dog 35-127.

Period No.	Urine			Acetone bodies		Plasma protein	Plasma volume	D:N ratio
	Total N	Urea N + NH <sub>3</sub> -N	Sugar	Blood	Urine			
	gm.	per cent	gm.	mg. per cent	gm.	gm. per cent	cc.	
1	3.49	77	11.11	59	0.59	5.58	432	3.18
Plasma by vein				19.08 gm. protein				
2	3.77	76	11.69	58	0.52	5.96		3.10
3	3.28	77	9.55	47	0.46	6.18		2.91
4	2.76	75	7.24	55	0.29			2.62
5	2.39	75	7.46	89	0.50	6.42	395	3.12

there is no evidence that this protein has been catabolized in the usual manner, since no excess nitrogen or sugar is recovered.

Control experiments were conducted in which 300 mg. of anhydrous glucose in 300 cc. of Locke's solution were injected. These amounts approximate the amount of blood sugar and the volume of plasma that had been injected. The experiments were performed in order to learn what effects the small amount of sugar and the volume of injected fluid might have on the acetone bodies.

Dog 35-15 had been used in the previous experiments and Table V records the data obtained. There is a slight decrease in

protein percentage in the circulating plasma, as would be expected in a fasting animal. The urine nitrogen and sugar progressively decrease. Following the injection of the sugar and Locke's

TABLE V  
*Glucose and Locke's Solution by Vein*

Dog 35-15.

Period No.	Urine		Acetone bodies		Plasma protein	D:N ratio
	Total N	Sugar	Blood	Urine		
	gm.	gm.	mg. per cent	gm.	gm. per cent	
1	4.54	19.86	101	3.34	6.34	4.38
2	4.59	16.73	101	3.77		3.64
3	4.40	16.02	130	3.76		3.63

By vein 300 mg. anhydrous glucose in 300 cc. Locke's solution

4	3.07	14.23	71	1.65		4.64
5	2.83	9.57	78	0.71		3.38
6	3.05	9.4	91	0.55	5.75	3.12

TABLE VI  
*Glucose and Locke's Solution by Vein*

Dog 35-13.

Period No.	Urine		Acetone bodies		Plasma protein	D:N ratio
	Total N	Sugar	Blood	Urine		
	gm.	gm.	mg. per cent	gm.	gm. per cent	
1	4.46	17.39	57	2.78	5.99	3.89
2	4.52	17.71	82	2.63		3.91
3	4.31	17.62	79	2.66		4.08
4	4.38	16.42	74	1.98		3.74

By vein 300 mg. anhydrous glucose in 300 cc. Locke's solution

5	4.01	17.66	75	2.28		4.41
6	4.22	16.13	71	0.88	5.80	3.82

solution the acetone bodies of both blood and urine decrease and remain low, particularly in the urine. The weight fell from 15.1 to 14.3 kilos.

A control experiment was performed on Dog 35-13 also, and

there was but little change in the circulating plasma protein percentage (Table VI). The urine nitrogen following the injection of sugar in the Locke's solution is slightly less in amount than during the fore-basal periods, but the urine sugar is on the same general level. The increase in urine acetone bodies at the end of the injection period may be the result of a flushing out by the fluid. The weight decreased from 15.2 to 14.5 kilos.

#### DISCUSSION

Previous experiments in this laboratory have shown that the dog has protein stores that it can draw upon when there is a demand for plasma protein formation. These stores become depleted upon fasting of the animal (2, 7-9). Luck (5) has given evidence for liver storage of proteins related to the diet and Addis (1) and his associates have reported that when rats are fasted there is prompt decrease in the protein content of the liver. In our experiments the dogs were fasting and also were under the additional strain of being diabetic owing to phlorhizin. During the 4 or 5 days prior to the injection of the plasma protein there undoubtedly was utilization of reserve protein stores with partial depletion. Consequently the body was in a state where it would welcome any protein introduced and utilize it to the best of its ability. The data indicate that the injected protein very promptly disappeared from the blood stream. No protein was lost in the urine. However, there was no increase in urinary nitrogen or sugar as there was when the plasma protein was fed.

What happens to this protein when it leaves the blood stream? It might be argued that it was removed as such and stored in the body without alteration. The body cells vary as regards their protein make-up and it seems unlikely that plasma protein could be taken in by the cells to form their particular types of protein without its first undergoing alteration.

In these experiments there is evidence of nitrogen conservation following the injection of the plasma protein. It seems that the body economy is altered as the result of receiving this protein material. In other experiments performed in this laboratory the evidence all points to actual utilization of the protein (2, 9, 3). When dogs are fed a protein-free diet and are given plasma by vein over a 2 week period, it is possible to maintain them

approximately in a state of nitrogen equilibrium. There is no loss of protein in the urine and no excess nitrogen is eliminated subsequently.

This utilization of the injected plasma protein without the elimination of any excess nitrogen or sugar points to a different metabolic mechanism on the part of the body. When the protein is fed, amino acids result from the complete digestion of the injected material. However, when injected, such breaking down of the protein does not occur, as no sugar is formed. We suggest the following mechanism occurs. The plasma protein is removed from the blood and then undergoes slight catabolic change with formation of large aggregates of amino acids and these are then reassembled by the cells to form their own particular protein matrix.

It is reasonable to believe that it is in this manner that protein exchange occurs within the body rather than that protein must be always completely catabolized and the desired type of protein then be elaborated from the amino acids.

#### SUMMARY

When phlorhizinized dogs are fed plasma protein, it is digested with conversion of part of it to sugar. Ketosis decreases as the result of the sugar formation.

When phlorhizinized dogs receive plasma protein by vein, the injected protein promptly disappears from the blood stream. No protein is lost and there is no excess elimination of nitrogen or sugar in the urine.

There is some decrease in the ketosis following injection of plasma protein and the dogs are clinically improved.

There is evidence of nitrogen conservation by the body following the injection of plasma protein.

The metabolism of protein when fed is different than when it is injected. It is suggested that there is partial catabolism of the injected protein with reassembly of the large aggregates formed by the cells to form their own peculiar type of protein.

This partial catabolism with reassembly of the large aggregates may be the method of protein interchange within the body rather than a complete catabolism to amino acids with subsequent re-synthesis to protein.



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## DETERMINATION OF CEPHALIN IN PHOSPHOLIPIDS BY THE ESTIMATION OF CHOLINE\*

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There are three types of phospholipids recognized at the present time, lecithin, cephalin, and sphingomyelin. The biochemical importance of these substances as a group is well established (1). Because of the great difficulty in quantitatively differentiating them (2), little is known of the biological rôle of the individual phospholipids.

During a series of studies (3-6) in this laboratory on the lipids of erythrocytes and plasma of blood dyscrasias it seemed significant to determine whether the differences noted in the phospholipids were due to parallel changes in the individual types constituting the phospholipid mixture, or whether the fluctuations were due to one particular type of phospholipid.

Attempts to determine the cephalin by the amino nitrogen method verified the finding of Van Slyke and coworkers (7), that the determination of the cephalin by this method cannot be made because of contaminating nitrogen compounds. A method was developed, based upon the relative solubilities of lecithin and cephalin in absolute alcohol (8). The results obtained with this method exhibited striking consistencies in the absolute alcohol-soluble phospholipids of erythrocytes and plasma and indicated the presence of larger amounts of cephalin in the erythrocyte (3, 4, 8).

\* A preliminary report of this investigation was presented before the Division of Biological Chemistry at the Ninety-fourth meeting of the American Chemical Society at Rochester, New York, September 9, 1937.

† Some of the data have been taken from the dissertation submitted by Ira Avrin in partial fulfilment of the requirements for the Degree of Master of Science in the Graduate School, Wayne University, Detroit.

Recently, Beattie (9), in England, published a microchemical method for the determination of choline. Inasmuch as cephalin contains no choline, and the choline content of lecithin and sphingomyelin is approximately equal, this method offered the possibility of estimating quantitatively the cephalin content of small amounts of phospholipid.

#### EXPERIMENTAL

Determination of the choline content of standard choline solutions demonstrated that the method was accurate within the limits stated by Beattie (9). Attempts were made to find a suitable means of hydrolyzing small amounts of phospholipid to liberate the choline quantitatively. Hydrolysis with hydrochloric acid proved difficult to control with small quantities, while the use of sodium hydroxide gave incomplete recoveries of choline. On the basis of several reports in the literature (10) that barium hydroxide readily liberates the choline from lecithin and sphingomyelin the effect of barium hydroxide on standard choline solutions was studied. Amounts of choline hydrochloride, ranging from 0.3 to 2.0 mg., were carried through the barium hydroxide hydrolysis procedure and determined as outlined under the method. The quantitative recovery of the choline obtained indicated that hydrolysis with barium hydroxide has little or no effect in the determination of choline.

The choline content of a standard ethyl ether solution of egg phospholipid, prepared according to the procedure of Bloor (11) for isolating blood and tissue phospholipids, was determined after hydrolysis with barium hydroxide. This solution, which contained 4.1 mg. of phospholipid per cc. determined by the Bloor oxidative titrimetric technique (11), had an average of 0.62 mg. of choline hydrochloride per cc. (15.1 per cent). Known amounts of choline hydrochloride added to this solution were recovered quantitatively. In order to determine the choline content of blood phospholipids, the plasma, erythrocytes, and stroma, from which the phospholipids were to be isolated, were extracted with alcohol-ether (3:1) as specified by Bloor (12). The alcohol-ether extract was then evaporated, *in vacuo*, below 50° in a stream of nitrogen and the lipids transferred to a 50 cc. centrifuge tube by three extractions with boiling petroleum ether.

It was found necessary to use this method of evaporating the

alcohol-ether extract, as it has been the experience of several investigators (13-15), as well as our own, that evaporation on the steam bath results in low yields of phospholipid. Apparently the phospholipids are decomposed or oxidized to the extent of being partially insoluble in petroleum ether when the alcohol-ether extract is evaporated in air. Recently, Bloor (16) has reported that a modification of the original procedure, which prevents oxidation of the phospholipids during the evaporation of the alcohol-ether extract, has been used in his laboratory for some years.

In this laboratory, various methods of evaporating the alcohol-ether extract and their effect on the subsequent recovery of the phospholipids by petroleum ether have been studied extensively (17). Alcohol-ether extracts of plasma, erythrocytes, and erythrocyte stroma, varying from 25 to 200 cc. in volume, were evaporated under different conditions. The small quantities of suspended residue which were insoluble in petroleum ether were separated by centrifugation and washed several times, the wash solutions being combined with the original petroleum ether solution. Exclusion of air during evaporation increased the yield of petroleum ether phosphorus from 5 to 22 per cent. Evaporation under nitrogen at reduced pressure and below 50° resulted in average recoveries of 80, 90, and 93 per cent of the alcohol-ether phosphorus by petroleum ether, of plasma, erythrocytes, and erythrocyte stroma respectively. Furthermore, precipitation and determination of the phospholipids of the petroleum ether solution according to the Bloor technique (11) gave values comparable to those calculated from the petroleum ether phosphorus with the factor 23.54 (13). Evaporation *in vacuo* under nitrogen below 50°, essentially the procedure of Man (14), was adopted for preparing the petroleum ether solution of the phospholipids from the alcohol-ether extract.

The phospholipids were precipitated from the petroleum ether according to the method of Bloor (11) and redissolved in moist ethyl ether.

#### *Method of Determining Choline Content of Phospholipids*

An aliquot of the ether solution of the phospholipids, as prepared above, is taken for the determination of the phospholipid by the oxidative titrimetric procedure (11). To a second aliquot

in a 125 cc. Erlenmeyer flask are added 15 cc. of 95 per cent ethyl alcohol and 1 cc. of a saturated solution of barium hydroxide. The flask is placed on the steam bath and the contents evaporated to dryness, the last traces of alcohol being removed with a stream of air. Caution is necessary at the end of the evaporation to avoid overheating the residue. 0.6 cc. of normal hydrochloric acid is added to the flask and the acidified residue extracted with three successive 10 cc. portions of boiling petroleum ether. The contents of the flask are then transferred to a 10 cc. test-tube with three successive 2 cc. portions of distilled water, making the final volume in the test-tube approximately 6 cc. To the solution in the test-tube is added 1 cc. of a freshly prepared, saturated solution of Reinecke salt<sup>1</sup> in water, and after the material has stood for 10 minutes or more to allow for complete precipitation of the choline, the determination is carried out exactly as specified by Beattie (9).

#### DISCUSSION

In the calculation of the percentage choline content of the phospholipids it is necessary to utilize an average molecular weight for the fatty acids which they contain. Choline hydrochloride has a molecular weight of 157.6. Stearyl oleyl lecithin has a molecular weight of 805.7 and would therefore have an equivalent of 19.56 per cent of choline hydrochloride. Palmityl oleyl lecithin has a molecular weight of 777.7 and would have an equivalent of 20.27 per cent choline hydrochloride. Sphingomyelin, on the basis of the figures of Bull (10) for the percentage composition of fatty acids, *i.e.* 57 per cent stearic acid, 25 per cent lignoceric acid, and 18 per cent nervonic acid, has a molecular weight of 784.5 and would have the equivalent of 20.09 per cent choline hydrochloride. It has been assumed, therefore, that 20.0 per cent represents a fair average for the choline content of lecithin and sphingomyelin, calculated as the hydrochloride, and this value was used to calculate the cephalin present. The formula  $100 - 5X = Y$ , where  $X$  is the percentage of choline hydrochloride and  $Y$  is the percentage of cephalin, is used for this calculation.

<sup>1</sup> The Reinecke salt was purchased from Eimer and Amend, New York.

The determination of the phospholipid phosphorus permits a more direct calculation of the cephalin and eliminates the use of an average value for the molecular weight of the fatty acids present. Cephalin contains no choline, whereas both lecithin and sphingomyelin contain 1 molecule of choline for each molecule of phosphorus. If the ratio of equivalents of choline to phosphorus

TABLE I

*Cephalin Content of Blood Phospholipids Calculated from Percentage Choline (A) and from Ratio of Choline to Phosphorus (B)*

	Sample No.	Plasma				Erythrocytes			
		Choline HCl	Ceph- alin (A)	Ratio of equiva- lents, choline HCl : P	Ceph- alin (B)	Choline HCl	Ceph- alin (A)	Ratio of equiva- lents, choline HCl : P	Ceph- alin (B)
		per cent	per cent		per cent	per cent	per cent		per cent
Beef.....	1	18.2	9.0			9.6	52.0		
" .....	2	15.9	20.5			8.6	57.0		
" .....	3	11.7	41.5			7.8	61.0		
" .....	4	12.6	37.0	0.55	45	6.9	65.5	0.33	67
" .....	6	15.2	24.0	0.71	29	8.8	56.0	0.42	58
Chicken.....		16.2	19.0	0.75	25	14.3	28.5	0.68	32
Human.....	1	15.5	22.5			8.9	55.5		
" .....	2	13.9	30.5			7.5	62.5		
" .....	3	15.3	23.5	0.62	38	8.3	58.5	0.37	63
" .....	4	11.9	40.5	0.46	54	9.4	53.0	0.41	59
Hemophilia..	1	18.3	8.5			10.1	49.5		
" .....	2	14.8	26.0			7.9	60.5		
Hemolytic icterus.....		16.6	17.0	0.75	25	9.4	53.0	0.42	58
Lipemia.....	1	17.7	11.5	1.01	0	8.4	58.0	0.40	60
" .....	2*	18.6	7.0	0.85	15	9.4	53.0	0.51	49
" .....	3†	17.2	14.0	0.77	23	6.4	68.0	0.30	70

\* From the same subject after 3 days on a low fat diet.

† From the same subject after 3 days on a high fat diet.

were 1:1, there would be present 100 per cent lecithin and sphingomyelin, and no cephalin. If the ratio were 0.8:1, there would be 80 per cent lecithin and sphingomyelin and 20 per cent cephalin, etc.

The determination of the phospholipid phosphorus requires very little additional material and was carried out by the gaso-

metric method of Kirk (18) where sufficient sample was available (Tables I and II). The choline content of the phospholipids of plasma and erythrocytes with the cephalin content calculated therefrom, together with a comparison of the cephalin calculated from the ratio of equivalents of choline to equivalents of phosphorus, is presented in Table I. The cephalin values, calcu-

TABLE II  
*Cephalin Content of Stroma Phospholipid Calculated from Percentage Choline (A) and from Ratio of Choline to Phosphorus (B)*

Stroma sample	Sample No.	Choline HCl	Cephalin (A)	Ratio of equivalents, choline HCl : P	Cephalin (B)
		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>
Beef	2	9.1	54.5	0.49	51
	5	10.1	50.0	0.44	56
	19	8.3	58.5	0.38	62
	20	6.6	67.0	0.44	56
	21	8.4	58.0		
	23	8.2	59.0		
	24	10.2	49.0	0.53	47
	29	8.7	56.5	0.44	56
	30	8.5	57.5	0.33	67
	31	9.1	54.5	0.40	60
Sheep	8	7.6	62.0	0.37	63
	9	10.2	49.0	0.49	51
	11	7.5	62.5	0.35	65
	12	8.0	60.0		
	26	9.5	52.5	0.44	56
Human	25	5.1	74.5	0.24	76
	32	10.3	48.5	0.47	53
Horse	22	9.4	53.0	0.64	36
Chicken	35	14.6	27.0	0.53	47
Turkey	33	11.9	40.5	0.46	54

lated by the two methods, are in much better agreement in the erythrocytes than in the plasma. The percentage content of cephalin in the phospholipids of the plasma is in most cases less than 50 per cent and in some instances less than 10 per cent. The cephalin content of the phospholipids of the erythrocytes is over 50 per cent with the exception of the avian cells. Moreover,

the erythrocyte phospholipids appear to have a more constant and uniform content of cephalin than those of the plasma.

Similarly, Table II gives values for the phospholipids of various samples of erythrocyte stroma. In most cases there is very close agreement between the cephalin values calculated by the two methods. The percentage of cephalin in the stroma phospholipid is comparable to that found in the phospholipids of the erythrocytes and varies within a small range.

It appears that, with the exception of avian blood, the major portion of the phospholipids of the erythrocytes and red blood cell stroma is cephalin; whereas in the plasma it is the minor fraction of the phospholipids. This is in agreement with the view (1) that cephalin functions mainly as a structural phospholipid and lecithin functions in great part as a metabolic phospholipid.

#### SUMMARY

A method is proposed whereby the cephalin content of small quantities of blood and tissue phospholipids can be calculated from the determination of the choline and phosphorus present.

In a series of normal and pathological blood samples the cephalin content of the plasma phospholipids, calculated from the percentage choline content, averaged 22 per cent within the range of 7 to 41.5 per cent. The cephalin content, calculated from the ratio between the equivalents of choline and phosphorus, averaged 28 per cent within the range 0 to 54 per cent.

From the same samples the corresponding values for the erythrocytes were an average of 56 per cent within a range of 28.5 to 68 per cent, and an average of 57 per cent within a range of 32 to 70 per cent.

The cephalin content of phospholipids in red blood cell stromata prepared from various species, calculated from the percentage choline content, averaged 55 per cent within the range 27 to 74.5 per cent; calculated from the ratio between the equivalents of choline and phosphorus, averaged 56 per cent within the range of 36 to 76 per cent.

The phospholipid, cephalin, appears to be a minor component of plasma but a major component of the intact erythrocytes and red blood cell stroma. This conclusion may be taken to demon-



strate the importance of cephalin as a structural type of phospholipid.

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## ISOLATION OF CREATININE FROM SERUM ULTRAFILTRATES\*

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In earlier studies (1, 2) it was shown that creatinine originating from a source other than creatine could be isolated from normal beef and dog blood. Its origin from the preformed chromogenic substance of the whole blood filtrates could, however, not be proved; in fact there were definite indications to the contrary (2). From the blood of dogs and human subjects in nephritis and other types of retention, large amounts of creatinine were isolated, and its origin from the substance usually determined as creatinine was quite evident.

Direct precipitation of apparent creatinine from an ultrafiltrate of normal serum would seem to be the least involved method of obtaining the substance or its chromogenic constituent for chemical study. One of us found that this occurred if the ultrafiltrates were refrigerated after addition of suitable amounts of picric acid and rubidium chloride. Further experiments indicated that the chromogenic substance which precipitated originated from the preformed chromogenic substance of the ultrafiltrates, not from creatine or some other non-chromogenic substance (3). In the present study we have prepared picrate precipitates in adequate amounts from sera of several species, and decomposed them at room temperature with normal hydrochloric acid. By removing picric acid with ether, solutions of the chromogenic substance were obtained which had neither been heated nor submitted to other drastic procedures, although considerable time was involved owing to the scale of operation. The compound

\* A preliminary report of our findings was made at the Thirty-first annual meeting of the American Society of Biological Chemists, at Memphis, April 22, 1937 (*J. Biol. Chem.*, 119, p. xxxvi (1937)).

present in these solutions gave the same creatinine value whether the picrate or the dinitrobenzoate method of analysis was used. We then prepared characteristic derivatives of creatinine from it and analyzed them. Throughout the procedure tests were carried out to show that no creatinine appeared from creatine or substances simulating it. The picrate precipitate, like that from urine (4), is complex, but the substances present are not very troublesome.

#### EXPERIMENTAL

##### *Isolation of Creatinine from Ultrafiltrates*

Fresh blood was collected in 250 cc. centrifuge bottles, where it was allowed to clot. The serum was obtained from 1 to 4 hours after the animals were bled. Ultrafiltrates were prepared at the rate of 100 cc. per hour by the method previously described (3). Four lots of picrate precipitate—two from beef serum and two from pig serum—were prepared. Details concerning Lot 1 are shown in Table I.

*Precipitation*—To each 100 cc. of ultrafiltrate from beef serum we added 1.7 gm. of picric acid, stirred to solution, and with rapid stirring added 0.3 cc. of 10 per cent rubidium chloride. After 1 day of refrigeration at 5° a small, pale yellow, non-chromogenic precipitate had appeared. The chromogenic precipitate appeared on succeeding days. Ultrafiltrates of pig serum or dog serum yielded neither of these precipitates for many days when they were treated in an identical manner, a difficulty not encountered in precipitating added creatinine (3). One of our lots of picrate precipitate from ultrafiltrates of pig serum was prepared by adding to each 100 cc. of ultrafiltrate 2 cc. of a lithium carbonate solution containing 1 mg. of uric acid per cc., then proceeding exactly as with ultrafiltrates of beef serum. This modification was based upon the fact that uric acid proved to be a noteworthy constituent of the precipitates from beef serum ultrafiltrates. The other lot was prepared by the less desirable modification of doubling the quantity of rubidium chloride. A large excess of rubidium picrate precipitated, and made decomposition of this lot a tedious procedure.

*Collection*—As soon as precipitation had reached profitable limits, each lot of ultrafiltrate was filtered, at room temperature,

through a Jena sintered glass filter tube. As shown in Table I, ultrafiltrates of beef serum were usually refrigerated 4 to 5 days after addition of picric acid and rubidium chloride; those of pig serum required 8 to 10 days for the precipitation despite the modifications already mentioned. Successive lots of precipitate were collected in the same Jena glass filter tube until the desired quantity from each source was at hand. The weight of these precipitates, after being washed with alcohol-ether mixture and dried

TABLE I

*Details of Preparing, from Beef Serum Ultrafiltrates, Picrate Precipitate Estimated to Contain 35.73 Mg. of Creatinine*

Ultrafiltrate No.	Date	Volume	Creatinine content		Creatinine in ppt.* (calculated)	Time required for precipitation
			Before precipitation	After precipitation		
	1936	cc.	mg. per 100 cc.	mg. per 100 cc.	mg.	days
1	Sept. 11	640	2.21	0.72	9.48	3
2	" 18	190	2.15	0.80	2.53	3
3	" 18	190	2.35	0.92	2.63	4
4	" 19	266	2.20	1.22	2.53	5
5	" 19	130	1.75	0.52	1.55	4
6	" 24	365	2.12	0.72	5.08	4
7	" 24	264	2.07	0.75	3.43	4
8	" 28	232	2.15	0.70	3.31	5
9	" 28	212	2.25	0.72	3.15	5
10	Oct. 3	178	1.87	0.70	2.04	7
Total.....		2667			35.73	
Average.....			2.11	0.78		4.4

\* Corrected for samples withdrawn for analysis.

*in vacuo*, was 430 and 443 mg. in the case of Lots 1 and 3 respectively. Lot 4, prepared by doubling the amount of rubidium chloride, weighed 2.850 gm.

*Decomposition*—Lot 1 of picrate from beef serum was dissolved in hot water. Picric acid was then removed with ether after slight acidification. The Lots 2, 3, and 4 were all decomposed at room temperature by allowing N hydrochloric acid to percolate through the filter tube without suction. Successive

10 cc. and finally 5 cc. portions of the acid were used, 50 to 60 cc. being required for complete extraction. The amounts of creatinine extracted in this way compared well with estimates based on determinations before and after precipitation, as shown in Table II. This confirms the view that the creatinine was derived from the preformed chromogenic substance of the ultra-filtrates. Picric acid was removed by repeated extraction with ether, the latter in turn by aerating and applying a vacuum. Some unfortunate losses occurred at this point.

TABLE II

*Amount of Creatinine Obtained by Decomposing Four Lots of Picrate Precipitate; Also Agreement of Picrate and Dinitrobenzoate Methods of Analysis*

Stage of isolation and method of analysis	Creatinine content			
	Lot 1, beef serum	Lot 2, beef serum	Lot 3, pig serum	Lot 4, pig serum
	mg.	mg.	mg.	mg.
Picrate ppt., estimated by picrate method as in Table I.....	34.8	37.8	41.8	40.4
N HCl extract of picrate ppt.; picrate method.....	33.8	35.5	40.4	38.4
N HCl extract after removal of picric acid; picrate method.....	27.4	25.3	31.7	35.2
Same solution; dinitrobenzoate method.....	27.0	25.4	31.7	35.1

*Analysis by Picrate and Dinitrobenzoate Methods*—Since the solutions contained 0.5 mg. of creatinine per cc., or even more, we used the 3,5-dinitrobenzoate method for urine, as described by Benedict and Behre (5). A suitable creatinine standard and 0.5 cc. of the hydrochloric acid solution were both neutralized with sodium hydroxide. The volume in each tube was made to 2 cc. with water. 3 cc. of a 1 per cent alcoholic solution of 3,5-dinitrobenzoic acid and 1 cc. of 6 per cent sodium hydroxide were added. 10 to 12 minutes later the solutions were diluted with 10 cc. of distilled water. Within 8 minutes thereafter colorimetric comparison was made. Excellent agreement with analyses made by the picrate method is shown in Table II.

*Conversion to Creatinine Zinc Chloride and Creatinine Potassium*

*Picrate*—After evaporating the hydrochloric acid solutions to dryness on a water bath, we extracted creatinine hydrochloride with absolute alcohol. Tests of the extracts and the alcohol-insoluble residue showed that no creatinine was formed during evaporation and that extraction was complete. The alcohol solutions were evaporated, and the creatinine was converted to the zinc chloride in exactly the manner described in an earlier paper (1). The recoveries were 91.4, 94.5, 95.4, and 92.0 per cent in Lots 1 to 4. The crystals formed on the walls of the tube like creatinine zinc chloride, and were microscopically identical with it. But an amorphous precipitate which contained nitrogen was also present; consequently purification was effected

TABLE III  
*Analyses of Creatinine Potassium Picrate Obtained from Beef and Pig Serum Ultrafiltrates*

Lot No. and source of picrate	Weight of sample <i>mg.</i>	Creatinine content	
		<i>mg.</i>	<i>per cent</i>
1. Beef	8.693	1.616	18.59
2. "	9.327	1.698	18.20
3. Pig	9.766	1.804	18.47
4. "	8.591	1.581	18.40
Average.....			18.41

by dissolving the entire precipitate in hot 1.2 per cent picric acid and precipitating creatinine as the double picrate by adding potassium chloride and cooling the solution. The losses are almost negligible if the excess of potassium (1) is properly regulated. After at least three recrystallizations the double picrates were dried and analyzed.

*Analysis of Double Picrates*—The technique used in the first isolations (1) was again employed, with this refinement, that the samples of picrate were weighed with a microchemical balance<sup>1</sup> sensitive to 0.001 mg. The weighed samples were dissolved in hot 1.2 per cent picric acid, transferred, and made up to 100 cc. with the same solvent. Each solution and two dilutions of it

<sup>1</sup> We are indebted to the Chemistry Department of Wayne University for the use of their microchemical balance.

were analyzed for creatinine by the picrate method. In each determination six readings were taken at 15 mm. and six at 20 mm. depth. The average results are recorded in Table III. The theoretical creatinine content of creatinine potassium picrate is 18.58 per cent.

Approximately 40 mg. lots of the double picrates were decomposed with hydrochloric acid and ether. The solutions were then analyzed for creatinine by the picrate and the dinitrobenzoate methods, and for nitrogen by digestion with sulfuric acid, sodium sulfate, and copper sulfate, followed by distillation with sodium hydroxide and titration of the ammonia with 0.01 N acid and

TABLE IV  
*Analyses of Picrate-Free Solution Obtained by Decomposing Isolated Creatinine Potassium Picrate*

Lot No. and source	Creatinine content of 2 cc.		Nitrogen content of 2 cc.	
	Picrate method	Dinitrobenzoate method	Amount	Per mg. creatinine
	mg.	mg.	mg.	mg.
1. Beef	1.75	1.74	0.654	0.374
2. "	1.79	1.80	0.667	0.372
3. Pig	1.78	1.76	0.640	0.362
4. "	1.40	1.40	0.528	0.377

alkali. Duplicates agreed to within 0.01 mg. of nitrogen. The amount of nitrogen per mg. of creatinine (the latter determined colorimetrically) was near the theoretical value of 0.372 mg., as shown in the last column of Table IV.

#### *Other Components of the Picrate Precipitate*

*Uric Acid*—Lot 1 of picrate from beef serum ultrafiltrates, as stated above, was dissolved in hot water. On cooling, a yellow precipitate formed which redissolved on heating, but a white crystalline substance remained. This was washed and weighed in a sintered glass crucible. Hot phosphate solution dissolved 26.0 mg. of the substance, leaving only a trace. The phosphate solution, analyzed colorimetrically by the Benedict-Franke method (6) contained 26.3 mg. of uric acid. Jaffe (4) also found uric acid in the picrate precipitate from human urine. It was

rather surprising that we obtained practically 1 mg. of uric acid per 100 cc. from ultrafiltrates of beef sera, which, according to evidence obtained with other types of filtrates, contain only traces of uric acid (7). Since we obtained no uric acid from pig serum or dog serum, the origin of our uric acid from the uric acid-ribose compound (8, 9), which is relatively abundant in the cells of beef blood, and of which little is present in pig blood or dog blood, appeared possible. We have not determined whether the enzymatic decomposition of this compound is so appreciable in the short time that the sera remained in contact with the clot.

*Unidentified Residues*—The hydrochloric acid solutions obtained by decomposing the picrate precipitate were evaporated, after the removal of picric acid. Alcohol extracts of the residues contained about 30 per cent more nitrogen than was required for the creatinine present. Part of this nitrogenous material precipitated when potassium acetate was added to neutralize the hydrochloride. We suspected the presence of basic amino acids, but tests for histidine were negative, and while the Sakaguchi reaction (10), to which arginine responds, was positive, the ninhydrin reaction was quite weak. The amount of material was too small for further study.

#### DISCUSSION

We have previously shown that a large part of the apparent creatinine in ultrafiltrates of human, dog, beef, and pig sera can be precipitated under conditions which differ greatly from those required for precipitation of added creatinine (3). In two species from which serum was available in unlimited quantities we have now completed the chain of evidence linking the precipitable fraction with creatinine. Theoretical as well as technical purposes were considered in devising the procedure, which begins with ultrafiltration and parallels the conventional methods of preparing creatinine from urine.

Regarding the technical side, ultrafiltrates can be prepared at the rate of 100 cc. per hour, a fairly rapid rate if one considers that this volume is equivalent to much larger volumes of ordinary filtrates. Initial concentration of filtrates, the use of adsorbents, or the introduction of mercury salts and hydrogen sulfide was avoided. Every solution or precipitate could be tested with al-



kaline picrate, so that the compound could be traced completely. At an early stage in the procedure the compound gave the same creatinine value by the dinitrobenzoate method as by the least specific of the picrate methods,<sup>2</sup> indicating the absence of substances yielding extraneous color. The liabilities of the procedure are clearly too great to permit the conclusion that the creatinine which was isolated must have been present as such in the ultrafiltrate, but there was no doubt concerning its origin from part of the apparent creatinine determined in the ultrafiltrates.

The very significant studies of Miller and Dubos (14) appeared in preliminary form while our study was in progress, and the details have just been published. These authors reach the conclusion that creatinine is present as such in human blood, the plasma containing about 1 mg. per 100 cc. While a comparative study has not been made, we anticipate that the fraction of the apparent creatinine which they destroy with their enzyme is the same as that which we precipitate.

The work of Goudsmit (15), on dogs under anesthesia, has supplied the essential evidence that the kidney removes significant amounts of the chromogenic substance present in blood. Without such evidence the presence of creatinine itself in blood would be of no significance from the standpoint of explaining its excretion. We have not been successful in carrying out our entire isolation procedure in the case of the dog, as we had hoped to do, in order to complete the evidence linking "chromogenic substance" with urinary creatinine in the same species.

The extraordinary difficulty of precipitating apparent creatinine, which was none the less ultrafiltrable, naturally led us to consider whether we were not dealing with creatinine in labile combination with another substance separated from it in our procedure, and possibly reabsorbed by the renal tubule. We have no final evidence on this point. The difficulty varies with

<sup>2</sup> The method was based on the original one of Folin (11). The standard and unknown solutions both contained 1.2 per cent of picric acid, and 0.5 cc. of 10 per cent sodium hydroxide was added to 10 cc. of each solution. In the later procedure of Folin and Wu (12) the picrate concentration is greatly reduced, and in the last modification Folin (13) reduced both the picrate and the alkali concentrations. For detection of substances simulating creatinine the least specific method is the method of choice.

the species. Although the average yield of creatinine from pig serum was almost identical with the yield from beef serum, the problem of precipitation was far greater. The addition of uric acid solved the technical problem fairly well in this case, but with dog serum various additions were made without success in devising a large scale procedure that was dependable enough for isolation work. We have as yet no explanation of these peculiarities. They are not encountered in precipitating added creatinine; hence we could not disregard them.

The amounts of creatinine which we isolated were quite significant. The initial picrate precipitates from twenty-one beef serum ultrafiltrates used in the present study contained the equivalent of 1.36 mg. of creatinine per 100 cc.; the average yield from thirty-two ultrafiltrates of pig serum was 1.28 mg. per 100 cc. About 77 per cent of these amounts (Table II) was still present when the comparison between picrate and dinitrobenzoate methods of analysis was made, some mechanical losses having occurred.

Linneweh (16) isolated and identified creatinine from beef blood by a modification of the Lloyd's reagent procedure described by one of us (2). In discussing the work of Gaebler and Keltch (1) he evidently overlooked the fact that experiments with added creatine were carried out, and that determinations were made before and after every procedure that might convert creatine to creatinine. Concerning identification, Linneweh remarks that betaine also gives a zinc chloride derivative. Gaebler and Keltch, however, isolated creatinine as creatinine potassium picrate, and analyzed this for colorimetric creatinine content per unit of weight and for non-picrate nitrogen in a colorimetrically determined sample. The compound was then converted to the zinc chloride derivative, and the nitrogen analyses repeated. Betaine and many other compounds fail in the first place to give a creatinine reaction by the method that was used. In the present study we have made the colorimetric analyses by two different methods.

Recently Zacherl (17) has reported isolations of both creatinine and creatine (after conversion to creatinine) from beef serum. Both Zacherl and Linneweh identified the creatinine derivatives which they isolated by elementary analysis. They conclude that the creatinine which was isolated was present in the blood as

such—not, however, on the basis of procedures less open to possible objection than those which we have employed.

#### SUMMARY

1. A large part of the apparent creatinine determined in beef and pig serum ultrafiltrates by a picrate method similar to Folin's original procedure can be isolated as creatinine. The average yield of creatinine was about 1 mg. per 100 cc. of ultrafiltrate in each of the two species.

2. Uric acid was found in the picrate precipitate from ultrafiltrates of beef serum; other nitrogenous substances were present in precipitates from the ultrafiltrates of beef and pig sera.

3. The difficulty of precipitating apparent creatinine from serum ultrafiltrates varies with the species, increasing progressively as beef, pig, and dog sera are used.

4. In connection with the observed differences between apparent creatinine and added creatinine in ultrafiltrates, only labile, and as yet unknown, creatinine compounds are considered.

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# OBSERVATIONS ON THE ALLEGED PRESENCE OF LIPID CHLORINE IN THE BLOOD AND TISSUES

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The assumption that all of the chlorine of the body is inorganic has been challenged at times (Hanke and Donovan, 1927; Morris and Morris, 1930; Peters and Man, 1934). Interference by lipids in the determination of chlorides has been recognized as a possible explanation of such results (Hanke, 1931; Sunderman and Williams, 1931; Drevon, 1935). However, Peters and Man showed that chlorine soluble in petroleum ether could be obtained by extraction of serum with a mixture of alcohol and ether. They were not able to extract chlorine directly with petroleum ether. We can find no reports of the direct extraction of chlorine from tissues by petroleum ether (*cf.* Cameron and Walton (1928)) nor by diethyl ether (*cf.* Hogartz (1931)).

Observations made while studying the metabolism of brominated lipids suggested to us that an explanation of the contradictory nature of these findings lay in the methods employed in extraction of the lipid fractions found to contain chloride. A portion of the bromine administered to an animal, or added as sodium bromide to solutions during extraction, could be recovered in the phospholipid fraction of the lipid extracts. Furthermore, analysis of such fractions has revealed the presence of chlorine (Tables I and II). The method of extraction is one commonly used, similar to that of Peters and Man: several extractions by warm alcohol-ether (Bloor), evaporation of this solution to dryness *in vacuo*, and extraction of the residue with petroleum ether. The report of lipid chlorine in the tissues might be explained by the assumption that chloride is bound by lipid material during or

after the evaporation of the alcohol-ether extract, thereby becoming soluble in petroleum ether. We use the term binding to represent the observed association of halides with the lipids, with no implication as to the mechanism causing it.

It is readily shown that the chlorine present in the petroleum ether solutions obtained following the alcohol-ether extraction of several tissues is substantially all in the acetone-precipitable fraction (Table I). This is true also of the lipid chlorine of human blood (Table II). The chlorine content of these tissue extracts is increased by addition of sodium chloride to the alcohol-ether solutions preceding evaporation (Tables I and II).

TABLE I  
*Lipid Chlorine of Several Tissues*

Source of lipid extract	Total	Precipitated by acetone
	mg.	mg.
Brain, 10 kilo dog.....	7.75	7.13
Same, in presence of 1 gm. NaCl*.....	50.0	49.0
293 gm. rat.....	8.3	8.2
Blood of Cat 3, per 100 ml.....	5.1	4.7
" " " 4, " 100 " .....	3.5	3.3

\* The chlorine content is calculated on the basis of the lipids of the entire brain. The experiment was carried out with aliquot amounts of extract and NaCl.

In order to refer the chloride binding to substances characterized better, lecithin and cephalin were isolated in fairly pure states. Lecithin was prepared from egg yolk by use of the cadmium chloride complex by the method of Levene and Rolf, as modified by Maltaner, and as given by Morrow and Sandstrom (1935). This product contained no detectable amount of cephalin but approximately 0.05 per cent of chlorine. Cephalin, prepared from calf brain according to the extraction method of Renall (1913) followed by numerous precipitations by alcohol and by acetone from petroleum ether solutions, was practically free from chlorine. When these preparations were introduced into alcohol-ether mixtures to which varying amounts of sodium chloride had been added (100 ml. of alcohol-ether plus 5 ml. of water containing the sodium chloride) with the usual evaporation *in vacuo*, extraction into petroleum

ether, and thorough centrifugation, amounts of chlorine were found present varying from 1.0 to 1.8 per cent of the lecithin and from 3 to 5 per cent of the cephalin used. In no case did cephalin bind more than 5 per cent of chlorine, which represents, approximately, 1 atom of chlorine per molecule. With the blood of five cats we found respectively 5.0, 4.0, 7.4, 5.1, and 3.5 mg. of lipid chlorine per 100 ml., amounts not too large to be bound by the phospholipids. Calculations, however, from values of Peters and Man for lipid chlorine and lipid phosphorus reveal that in normal serum the lipid chlorine was as high as 8 per cent, and in that of

TABLE II  
*Lipid Chlorine in Human Blood*

Subject	Total		Fraction 1, precipitated by acetone	Fraction 2, soluble in acetone.	Fraction 3, soluble in acetone and petroleum ether	Fraction 4, precipitated* by acetone from water emulsion	Total†
	mg. per cent	m.-eq. per l.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
G. D.	15.1	4.2	13.2	1.9			
H. C.	26.4	7.4	23.8	2.6			
	22.2	6.3	20.3	1.9	0.4	-	
F. S.	17.4	4.9	16.3	1.1	0.2		
P. W.	28.6	8.0	27.0	1.6	0.2	1.0	
E. N.	22.5	6.3	19.9	2.6		1.6	34.8
						0.8	33.4

\* Chlorine of precipitate obtained by adding acetone to water suspension of the acetone-precipitable fraction.

† 150 mg. of NaCl were added to the alcohol-ether-blood mixture during extraction.

pathological cases as high as 12 per cent of the phospholipids. As it seemed surprising that the phospholipids could bind this quantity of chlorine, the lipid chlorine of the whole blood of a number of normal individuals was determined.

5 ml. samples of freshly drawn blood were sprayed into solutions of 100 ml. of 95 per cent ethyl alcohol and 30 ml. of diethyl ether. These were warmed to boiling with rotation and filtered. The residues were reextracted with 30 ml. portions of hot alcohol. These solutions were evaporated to dryness and taken up in petroleum ether and centrifuged clear. The results for whole blood in

Table II agree with those of Peters and Man for whole blood and serum of normal individuals. About 90 per cent of the chlorine remained in the fraction (Fraction 1) precipitated by acetone. That the chlorine left in the acetone solution was no longer so soluble in petroleum ether was shown by evaporating the acetone solution to dryness *in vacuo* and extracting the residue into petroleum ether, which was then centrifuged over a drop of water. This petroleum ether solution (Fraction 3) was practically free of chlorine. When the acetone precipitate of the extract of 5 ml. of blood was emulsified with 1 ml. of water by rubbing in a small mortar, and 0.5 ml. of acetone was added with stirring, the principal portion of the phospholipids was obtained as a precipitate that formed gradually and could be centrifuged down. This precipitate contained little chlorine (Fraction 4), the major part remaining in the aqueous solution.

It was noted that when a solution of cephalin in petroleum ether was shaken with sodium iodide, qualitative tests showed the presence of much iodine in the petroleum ether layer (centrifuged clear). With sodium iodide, lecithin in petroleum ether yielded a gelatinous precipitate, and qualitative tests showed iodine in both precipitate and solution. Pure petroleum ether does not take up enough sodium iodide to give qualitative tests. These observations suggested a comparison of the binding of the three halides by cephalin and lecithin in petroleum ether solutions.

An excess, 0.50 gm., of sodium chloride and equivalent quantities of the bromide and iodide were shaken mechanically 24 hours in an atmosphere of nitrogen with 80 ml. portions of petroleum ether containing a known amount of phospholipid. The solutions were made to 100 ml. after centrifugation and aliquots taken for analysis (Table III). We can indicate the relative amounts of the halogens bound by cephalin as follows: iodide > bromide > chloride; and by lecithin: bromide > chloride. Iodide was not used with lecithin because of the formation of a precipitate. Whereas practically equivalent amounts of sodium and chlorine were bound, this was not the case with sodium and bromine, nor with sodium and iodine. As the presence of moisture might be responsible for this difference, undried c.p. halides having been used, a sample of completely dried sodium bromide was tried. Sodium and bromine were then present in practically equivalent though smaller amounts. When

petroleum ether saturated at 0° with ammonia was used as the solvent, the amount of sodium found became less than the amount of halide (Solutions 11 to 14). Apparently the ammonia displaced a portion of the sodium. The amounts of sodium bound in Solutions 3, 4, and 6 represent about 1 atom per molecule of cephalin.

TABLE III  
*Comparison of Binding of Ions by Lecithin and Cephalin*

Solution No.	Taken for experiment				Found in petroleum ether solution		
	Phospholipid*		Halide		Sodium	Halogen	Halogen
	mg.		gm.		m.-eq.	m.-eq.	per cent
1	310	Cephalin	0.50	NaCl	0.099	0.105	1.23
2	310	"	0.50	"	0.083	0.077	0.87
3	310	"	0.88	NaBr	0.43	0.24	6.2
4	310	"	0.88	"	0.39	0.19	4.9
5	310	"	1.28	NaI		0.62	25.0
6	310	"	1.28	"	0.38	0.66	27.0
7	270	Lecithin	0.50	NaCl			0.06†
8	270	"	0.50	"			0.06†
9	270	"	0.88	NaBr			1.04†
10	270	"	0.88	"			0.94†
11	200 Ca.	Cephalin‡	0.88	"	0.17	0.24	
12	200 "	" ‡	0.88	"	0.17	0.20	
13	200 "	" ‡	1.28	NaI	0.048	0.41	
14	200 "	" ‡	1.28	"	0.048	0.41	
15	128	"	0.88	NaBr§	0.050	0.047	2.9
16	128	"	0.88	" §	0.045	0.044	2.7
17	256	"	0.88	" §	0.107	0.107	3.3
18	256	"	0.88	" §	0.116	0.114	3.6

\* Dissolved in 80 ml. of petroleum ether. After being shaken with halide, the solution was made to 100 ml.

† 0.05 per cent chlorine present in lecithin was deducted.

‡ Petroleum ether, saturated with  $\text{NH}_3$ , was used as solvent.

§ Dried.

Iodide was the only ion bound in amounts larger than this. This is not explained.

Chlorine and the other halogens have been determined by Hald's modification of Patterson's microprocedure (1933). Sodium has been determined by Kramer and Gittleman's pyroantimonate procedure as modified by Eisenman and as given by Peters and Van



Slyke (1932). Petroleum ether (30–60°) has been purified by repeated washing with sulfuric acid and redistillation. Acetone has been dried by distillation from calcium chloride.

#### DISCUSSION

While we have been able to confirm the observations of Peters and Man that the petroleum ether extract of the residue left after the evaporation of the alcohol-ether extract of blood contains chloride, we feel that we have evidence that much or all of this lipid chlorine may be artifactual, being bound by phospholipid (*i.e.* acetone-precipitable) material during manipulation. The amount of lipid chlorine is increased by the presence of added inorganic chloride. Lecithin and cephalin combine with halides under the conditions of the extractions and when shaken with them in petroleum ether. Much chloride accompanies the material precipitated by acetone from petroleum ether, but little, that from water. The binding of the halides is presumably referable to the amphoteric nature of the phospholipids and their conceivable existence as zwitter ions. The amount of chloride associated with the lipids of human blood under the conditions used is apparently greater than can be attributed to lecithin and cephalin. More information concerning the nature of the lipids of human blood insoluble in acetone is necessary before their participation in the binding of chloride can be explained and evaluated.

We feel that for unquestioned demonstration of the presence of lipid chlorine in the blood or tissues interference by other lipids in the manner here shown must be ruled out. Furthermore, the appearance of halides in phospholipids after the administration of halogenated fatty acids cannot be taken as conclusive evidence of the incorporation of the substituted fatty acid in the phosphatides, as liberated inorganic halide may be carried by the lipid. It is interesting to note that Phillips, Halpin, and Hart (1935) have reported that most of the fluoride present in egg yolk is associated with the acetone-insoluble lipids following an alcohol extraction.

#### SUMMARY

The presence of chlorine in petroleum ether extracts of the material extractable from blood by a mixture of alcohol and ether,

as reported by Peters and Man, has been confirmed, and chlorine has been shown to be present in similar extracts of other tissues. Evidence is presented suggesting that this may be largely if not entirely artifactual, resulting from binding or entrainment of the halide during manipulation.

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## THE ISOLATION AND IDENTIFICATION OF THE ANTI-BLACK TONGUE FACTOR\*

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The preparation of concentrates of the antipellagra vitamin which were active in the prevention of pellagra-like symptoms in chicks and the cure of black tongue in dogs was described previously (1). In this paper we wish to report additional work on the purification of the vitamin which led to the demonstration of the activity of nicotinic acid and nicotinic acid amide in the cure of canine black tongue.

### EXPERIMENTAL

Dogs have been used exclusively for assay purposes. Several different breeds have been used but in each case the animals were brought to the laboratory shortly after weaning. They were given a complete diet for about 2 weeks, during which time they were kept under observation, and were then placed on the modified Goldberger diet<sup>1</sup> described previously (1). Of the entire group, two have been adult dogs, but the time required to produce black tongue in the older animals makes them rather unsatisfactory for this work. In order to reduce the loss of dogs to a minimum, the animals were used for the assays before severe

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<sup>1</sup> The casein was purified by washing crude casein with water eight times and then dissolving in ammonia and precipitating with hydrochloric acid.

symptoms developed. However, in every case, the supplement was not given until the dog showed drastic loss of weight and the early but definite symptoms of black tongue. Usually the animal refused its food for at least 2 days before the test material was administered. Many of the dogs were used for several assays. In each case the dog was continued on the basal ration until typical symptoms reappeared, at which time a new supplement was given. The extent of the growth response and the time required for the symptoms to reappear gave a fair indication of the quantitative potency of the material tested.

In our previous studies the purification had been carried through a final charcoal clarification step. Since the majority of the activity was recovered in the charcoal filtrate, it was concluded that the vitamin was not adsorbed on charcoal. It was planned, therefore, to continue to use this procedure for the preparation of relatively large amounts of the concentrate for isolation purposes. However, our original supply of charcoal was exhausted and our new material consisted of norit A (Pfanstiehl). When the activated norit was used, the filtrate proved inactive in the cure of black tongue (Fig. 1, Curves 1 and 2). When the same procedure was repeated with a sample of crude vegetable charcoal, the filtrate again contained a definite amount of activity (Fig. 1, Curves 3 and 4). It is evident that the concentrate which had been treated with this charcoal was almost as active as the untreated material. This observation emphasizes again the variable results which may be obtained with different samples of charcoal and that conclusions should be made only for the specific type used.

The use of the activated norit was continued because it was hoped that the adsorption and possible elution would offer a means of concentration of the vitamin. The following method was used. An aliquot of the concentrate carried down to the charcoal step according to the method described previously (1) equivalent to 400 gm. of liver extract<sup>2</sup> and containing about 6 gm. of solids was diluted to 200 cc. 4 gm. of norit A were added and the solution made to pH 3 with HCl. The suspension was stirred at 85° for 5 minutes, after which the norit was filtered off. The

<sup>2</sup> We are indebted to Dr. C. Nielsen of Abbott Laboratories and Dr. David Klein of The Wilson Laboratories, who supplied generous samples of liver extract.

norit removed approximately 1 gm. of the original solids. The norit was fed directly to the dogs in order to determine whether the total activity could be recovered. The norit showed activity, Fig. 1, Curves 5 and 6, but did not account for the entire potency of the extract before adsorption. If we assume that the vitamin was adsorbed more or less quantitatively without destruction, we may conclude that the dogs were able to elute only about one-third of the adsorbed vitamin.

The norit was treated with a number of reagents and combinations of reagents in an attempt to effect a quantitative removal of the vitamin. 4 gm. of norit carrying about 1 gm. of adsorbed solids equivalent to 400 gm. of liver extract were treated with 200 cc. of a mixture containing 2 parts of acetone, 1 part of water, and 1 part of pyridine. The mixture was heated to boiling for 3 minutes and the charcoal filtered off. This procedure was repeated twice. The eluate was concentrated to dryness *in vacuo* and the residue dissolved in water. About one-half of the solids adsorbed on the charcoal was recovered in the eluate. The activity of this eluate is shown in Fig. 1, Curves 7 and 8. From a rough estimate of the potency we may conclude that the elution was fairly quantitative.

Since this charcoal treatment aided materially in the removal of inert material and concentration of the active substance, it was made an additional step in our method. The following procedure was adopted as the routine method. Solutions containing 1 per cent solids were treated with sufficient norit A to give a 2 per cent suspension and the mixture adjusted to pH 4.0. The suspension was stirred for 1 hour at room temperature, after which the norit was filtered off and washed with distilled water. The norit was then added to a sufficient quantity of a mixture of 4 parts of methyl alcohol and 1 part of pyridine to form a 10 per cent suspension and the mixture was stirred for 15 minutes at room temperature. This procedure was repeated twice. The alcohol and pyridine were removed by vacuum distillation and the residue made up to a definite volume with water. This method of adsorption and elution was no more efficient than the method described above, but it was considerably more convenient. An assay of the concentrate carried through the above procedure is shown in Fig. 1, Curve 9.

Certain attempts to esterify the active compound were made

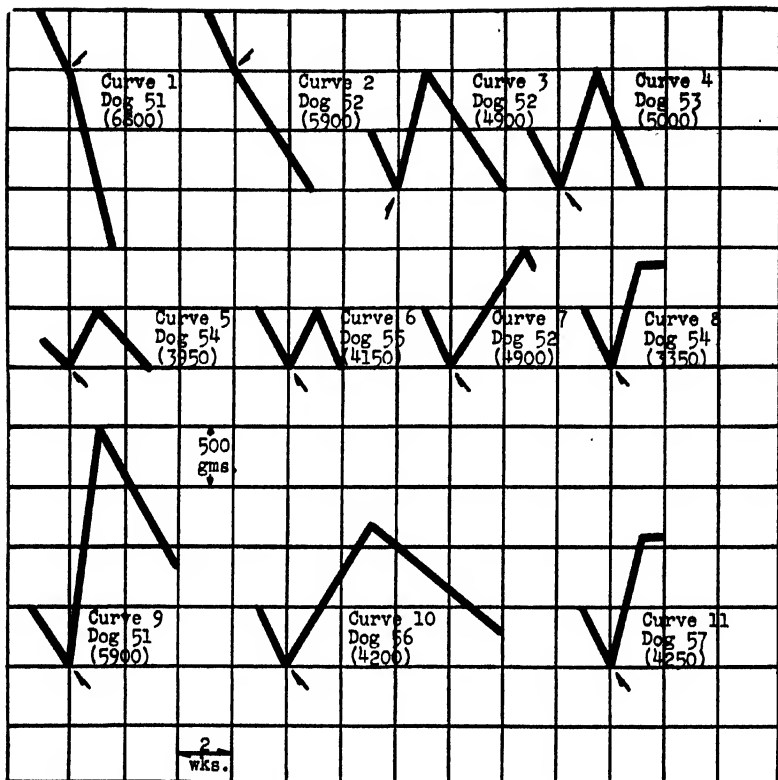


FIG. 1. Growth responses obtained in dogs maintained on basal black tongue diet when given various liver fractions. The arrow indicates the point of supplementation. The number in parentheses denotes the weight of the dog when the supplement was added. Curve 1, norit A filtrate fed in 10 cc. doses daily for 5 days. 1 cc.  $\cong$  80 gm. of fresh liver. Curve 2, norit A filtrate fed in 10 cc. doses daily for 8 days. 1 cc.  $\cong$  58 gm. of fresh liver. Curve 3, concentrate before treatment with a crude vegetable charcoal fed in 10 cc. doses daily for 4 days. 1 cc.  $\cong$  50 gm. of fresh liver. Curve 4, concentrate after treatment with a crude vegetable charcoal fed in 10 cc. doses daily for 4 days. 1 cc.  $\cong$  50 gm. of fresh liver. Curve 5, norit A with vitamin adsorbed fed in 1 gm. doses daily for 8 days. 1 gm.  $\cong$  200 gm. of fresh liver. Curve 6, norit A with vitamin adsorbed fed in 1 gm. doses daily for 8 days. 1 gm.  $\cong$  200 gm. of fresh liver. Curve 7, vitamin eluted from norit A with acetone, water, and pyridine fed in 10 cc. doses daily for 7 days. 1 cc.  $\cong$  40 gm. of fresh liver. Curve 8, vitamin eluted from norit A with acetone, water, and pyridine fed in 10 cc. doses daily for 7 days. 1 cc.  $\cong$  40 gm. of fresh liver. Curve 9, vitamin eluted from norit A with methyl alcohol and pyridine fed in 10 cc. doses daily for 4 days. 1 cc.  $\cong$  80 gm. of fresh liver. Curve 10, concentrate after treatment with NaOH fed in 10 cc. doses daily for 4 days. 1 cc.  $\cong$  60 gm. of fresh liver. Curve 11, concentrate extracted with acetone at pH 9.4 fed in 10 cc. doses daily for 4 days. 1 cc.  $\cong$  80 gm. of fresh liver.

at this point. In general these experiments were unsuccessful and the details will not be included in this paper. However, this work did lead to a more complete study of the stability of the vitamin to alkali, since it was necessary to hydrolyze the ester before feeding. The stability which was observed was truly remarkable. It is necessary to mention only one experiment, in which a concentrate containing 100 mg. of solids was treated with 50 cc. of 1 N sodium hydroxide and heated at 100° for 10 hours. The response obtained (Fig. 1, Curve 10) demonstrates that the activity is not reduced by this treatment. It was also found that the majority of the potency remained after refluxing a concentrate with 40 per cent hydrobromic acid.

After this stability was established, we were interested in the possibility of changing the solubility of the vitamin or its contaminants in organic solvents by forming sodium salts. At this stage we were fortunate in obtaining from Dr. H. W. Rhodehamel of Eli Lilly and Company concentrates prepared from both liver and liver extract according to our original procedure, except that the charcoal adsorption was omitted. The availability of these concentrates greatly facilitated our work and we wish to express our appreciation to Dr. Rhodehamel for this gift. An aliquot of a concentrate equivalent to 17 kilos of fresh liver was carried through the charcoal adsorption and elution, made to pH 9.5 with NaOH, and evaporated to dryness. The dry material was extracted with a total of 100 cc. of acetone. A determination of the solids showed that 0.8 gm. was left in the residue and 0.7 gm. was obtained in the extract. The activity of the acetone extract is shown in Fig. 1, Curve 11. It is evident from the response obtained and the equivalent amount fed that most of the activity was obtained in the acetone. This step gave no clear cut idea of the chemical properties of the compound but it did add another step which would remove about one-half of the total solids as inert material.

A considerable quantity of the liver extract concentrate was carried through the acetone extraction step. The final preparation gave no precipitate with alcoholic mercuric chloride or phosphotungstic acid. Several attempts were made to obtain crystals from different solvents without success. The material gave a negative test for sulfur and phosphorus. A nitrogen determination on the crude dried material showed about 10 per cent N.



While this work was in progress other studies in our laboratory (Frost and Elvehjem (2)) showed that nicotinic acid had some growth-stimulating effects in rats reared on certain purified diets. Upon comparison of the properties of nicotinic acid with those observed for the vitamin, we decided to test nicotinic acid itself

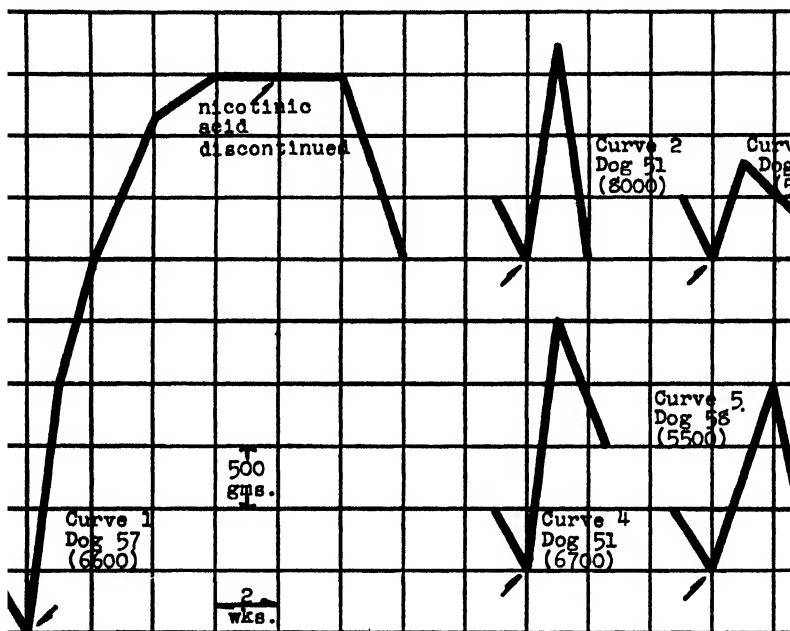


FIG. 2. Growth responses obtained in dogs maintained on basal black tongue diet when given nicotinic acid. The arrow indicates the point of supplementation. The number in parentheses denotes the weight of the dog when the supplement was added. Curve 1, 30 mg. of nicotinic acid every other day for 56 days. Curve 2, 40 mg. of synthetic nicotinic acid in one dose. Curve 3, 25 mg. of nicotinic acid in one dose. Curve 4, 60 mg. of nicotinic acid in three equal doses given on 3 successive days. Curve 5, 25 mg. of nicotinic acid in one dose.

on dogs. A dog showing all the symptoms of black tongue was given a single dose of 30 mg. of nicotinic acid (Eastman Kodak Company) and a phenomenal response was obtained. The appetite improved in a very short time, the mouth lesions disappeared in less than 2 days, and the growth response was very similar to that obtained with active concentrates. The responses ob-

tained in five different dogs are shown in Fig. 2. Dog 57 was kept on the basal diet plus 30 mg. of nicotinic acid every other day for 2 months, during which time the dog grew well and appeared normal in every way. Of the five dogs, four received commercial nicotinic acid, which is prepared by the oxidation of nicotine. Dog. 51 received nicotinic acid prepared from quinolinic acid merely to show that the commercial preparation did not carry impurities which might account for its activity.

The concentration of the vitamin from liver was continued through the aid of a molecular still. An aliquot of one of the

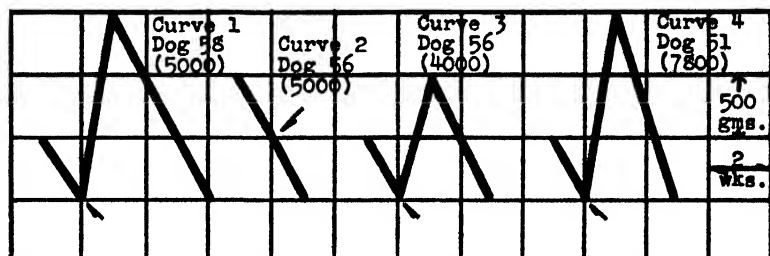


FIG. 3. Growth responses obtained in dogs maintained on basal black tongue diet when given nicotinic acid amide and the filtrate from which the amide had been removed. The arrow indicates the point of supplementation. The number in parentheses denotes the weight of the dog when the supplement was added. Curve 1, 108 mg. of a high vacuum distillate fed in one dose. 1 mg.  $\cong$  20 gm. of fresh liver. Curve 2, 58 mg. of the filtrate after nicotinic acid amide had been removed fed in one dose. Curve 3, 30 mg. of nicotinic acid amide in one dose. Curve 4, 50 mg. of nicotinic acid amide isolated from liver fed in one dose.

concentrates obtained from Dr. Rhodehamel equivalent to 2½ kilos of fresh liver was carried through the alkaline acetone extraction and 120 mg. of solids were obtained.

The entire material was placed in a molecular still, the condenser of which was cooled with solid CO<sub>2</sub>, and held at 160–165° and approximately 0.0001 mm. pressure for 3 hours. At the end of this time nearly all of the material had distilled, yielding a pale yellow, sticky solid. The entire distillate (108 mg.) was fed to a dog showing typical symptoms and an immediate response was obtained, Fig. 3, Curve 1.

Another aliquot equivalent to 10 kilos of liver was carried

through the acetone extraction and 450 mg. of solids were obtained. The entire preparation was distilled under similar conditions and the distillate was dissolved in about 4 cc. of alcohol and treated with an excess of saturated alcoholic  $\text{HgCl}_2$ . Almost immediately a white crystalline precipitate formed which was filtered off, washed, dissolved in dilute  $\text{HCl}$ , and decomposed with  $\text{H}_2\text{S}$ . When the resulting filtrate was concentrated to dryness, 175 mg. of long white needles were obtained. 50 mg. of these crystals when fed proved to be active, Fig. 3, Curve 4, while 58 mg. of the sirup resulting when the mercury was removed from the  $\text{HgCl}_2$  filtrate proved to be inactive, Fig. 3, Curve 2. The remainder of the crystals was recrystallized from alcohol-benzene, and melted at  $227\text{--}228^\circ$  (uncorrected). When mixed with nicotinic acid amide hydrochloride, the melting point was  $227\text{--}228^\circ$ .

$\text{C}_8\text{H}_6\text{ON}_2 \cdot \text{HCl}$ . Calculated. C 45.5, H 4.45, N 17.67

Found.<sup>3</sup> " 45.6, 45.9, H 4.9, 4.6, N 17.13, 16.97

The free base was prepared by removing  $\text{HCl}$  with  $\text{Ag}_2\text{O}$  from 16 mg. of the hydrochloride, and was crystallized from ethyl acetate. It melted at  $126\text{--}127^\circ$ , and when mixed with nicotinic acid amide, at  $127\text{--}127.5^\circ$ .

The chloroaurate was made by adding excess  $\text{AuCl}_3$  to a solution of 16 mg. of the hydrochloride in dilute  $\text{HCl}$ , and recrystallizing the yellow plates which formed from dilute  $\text{HCl}$ . The melting point was  $205^\circ$ , and was not changed when nicotinic acid amide chloroaurate was mixed with it. The compound did not decompose when melted.

$\text{C}_8\text{H}_6\text{ON}_2 \cdot \text{HAuCl}_4$ . Calculated, Au 42.6; found, Au 42.4

It was observed that the concentrate before distillation did not yield precipitates with alcoholic  $\text{HgCl}_2$  or with phosphotungstic acid, and for this reason it was at first thought that the nicotinic acid amide in the concentrate was not free, but that it perhaps was formed by decomposition during distillation of some more complex substance present in the concentrate. That this was not true, however, was shown by isolation of the amide from the concentrate before distillation. 25 mg. of the alkaline acetone ex-

<sup>3</sup> We wish to thank Mr. H. A. Campbell and Dr. K. P. Link for performing these analyses.

tract were dissolved in about 3 cc. of alcohol acidified with HCl and to this solution an excess of alcoholic  $\text{H}_2\text{PtCl}_6$  was added. The resulting precipitate was filtered off, washed with alcohol, dissolved in dilute HCl, and decomposed with  $\text{H}_2\text{S}$ . The filtrate from the  $\text{PtS}_2$  was concentrated to dryness, whereupon 13 mg. of crystals, melting at  $228^\circ$ , were obtained. From these crystals a chloroaurate was prepared which melted at  $205^\circ$ . It is thus apparent that 40 per cent of the concentrate before distillation could be isolated as free nicotinic acid amide in the form of its hydrochloride.

Nicotinic acid amide prepared from ethyl nicotinate was also fed to a few dogs to see whether it had the same activity as the amide isolated from liver. The results are given in Fig. 3, Curves 3 and 4. From the results obtained it appears that the activity of the synthetic amide and isolated amide is very similar. Nicotinic acid has been injected intramuscularly in two cases with results very similar to those obtained through oral administration. It is impossible at present to give any figures for the exact ratio of activity of the same compound when administered by mouth or by injection. It is interesting to mention that one dog showed such severe symptoms of black tongue that we did not expect to save the animal; however, a single dose of nicotinic acid injected intramuscularly brought about great improvement and it continued to improve upon additional oral feeding.

#### DISCUSSION

The results presented in this paper demonstrate conclusively that nicotinic acid and nicotinic acid amide are active in the cure and prevention of canine black tongue and that the activity of liver in the treatment of this disease is undoubtedly due to its content of nicotinic acid amide. Whether the majority of the nicotinic acid amide occurs in liver as such or in a more complex form cannot be answered at present. In any case the entire activity of liver may be correlated with its potential supply of nicotinic acid amide. Although it has been possible to isolate but a small amount of the total nicotinic acid amide in liver, we can estimate the original amount in a general way. We have used for isolation work liver extract preparations made in different laboratories but all preparations have shown about equal potency

when fed in amounts equivalent to the same quantity of fresh liver. A single dose of liver extract equivalent to 200 gm. of fresh liver fed to a dog suffering from black tongue cures the symptoms and gives a continued growth response for about 1 week. A single dose of 50 mg. of nicotinic acid amide gives a very similar response. If there has been no appreciable loss during the preparation of liver extract from liver, we may conclude that 100 gm. of fresh liver contain about 25 mg. of potential nicotinic acid amide.

In our final isolation 175 mg. of nicotinic acid amide crystals were isolated from liver extract equivalent to 10 kilos of fresh liver, which would contain about 2500 mg. of the amide. This is a recovery of only 5 per cent of the original material. The assays indicate that about 35 per cent of the potency was recovered in the concentrates carried up to the charcoal stage and that the recovery was reduced to 5 per cent after the acetone extraction step.

It is also possible from the few results given in this paper to estimate the nicotinic acid requirement of dogs. Since Dog 57 grew from 7 to 11 kilos in 5 weeks when receiving 30 mg. every other day, the intake would be about 1.5 mg. per kilo per day. The dogs weighing about 5 kilos grew for a little over a week when given one dose of 25 mg. and those weighing 8 to 9 kilos grew for about the same period when given 50 mg. of nicotinic acid. The requirement under the conditions in which we have worked may be tentatively set as 0.5 to 1.5 mg. per kilo per day.

Although there are no figures available for the amount of nicotinic acid in various foodstuffs, this compound and its derivatives are evidently rather widely distributed in nature. Nicotinic acid was first isolated from naturally occurring materials in 1912 by Suzuki, Shimamura, and Odake (3). Trigonelline, the methyl betaine of nicotinic acid, was isolated as early as 1885 by Jahns (4). In 1913 Funk (5) isolated nicotinic acid from the vitamin fraction of rice polishings. The activity of nicotinic acid in polyneuritis was very variable, but it was thought that it might be a decomposition product of the vitamin curing polyneuritis, or that some closely related compounds might show greater activity. These possibilities were also investigated by Williams (6) but

again variable and indeterminate results were obtained. Except for a paper by Szymańska and Funk (7), who attributed a food-sparing and weight-preserving action to nicotinic acid and the amide, very little interest was shown in the possible rôle of pyridine derivatives in living systems until the work of Warburg and Christian in 1935. They (8) characterized nicotinic acid amide as one of the hydrolysis products from the coenzyme which they had isolated from red corpuscles of horse blood. Kuhn and Vetter (9) isolated nicotinic acid amide from heart muscle and von Euler, Albers, and Schlenk (10) isolated the amide of nicotinic acid from cozymase.

This work gave new impetus to the application of these findings in the field of nutrition. Von Euler and Malmberg (11), using a ration very similar to the Sherman-Bourquin diet supplemented with a vitamin B<sub>1</sub> concentrate and flavin, found no growth response with nicotinic acid or nicotinic acid amide at a level of 1 mg. daily. However, the rats receiving the nicotinic acid lived longer than the controls. Funk and Funk (12) have reported recently that rats and pigeons on certain rations showed a larger food intake and better growth when given nicotinic acid and especially nicotinic acid amide. Frost and Elvehjem (2) obtained a very definite growth response in rats on purified rations through the addition of adenine nucleotides and nicotinic acid. It is also interesting to note that nicotinic acid and its derivatives play a part in the nutrition of lower organisms (Lwoff and Lwoff (13), Knight (14), and Mueller (15)). Thus nicotinic acid seems to have important functions in various types of organisms.

It is impossible to conclude definitely from the activity of nicotinic acid in canine black tongue that it will prove useful in the treatment of human pellagra. However, Spies has used nicotinic acid in four cases of classical pellagra and reports (personal communication) that the fiery red color associated with pellagrous dermatitis, stomatitis, and vaginitis improved promptly.

The use of nicotinic acid in the treatment of human pellagra brings up the possible toxicity of this compound. His in 1887 (16), Cohn in 1893 (17), and Abderhalden, Brahm, and Schittenhelm in 1909 (18) found that dogs fed 1 gm. daily of pyridine hydrochloride or pyridine acetate excreted equivalent amounts of methyl pyridinium hydroxide. The similar ability of humans to

methyrate pyridine is indicated from the work of Kutscher and Lohmann (19), who isolated small amounts of methyl pyridinium hydroxide from the urine of humans. The complete failure of rabbits to detoxicate pyridine was shown in the same work.

Cohn (17) isolated  $\alpha$ -pyridinuric acid, the dipeptide of picolinic acid and glycine, from the urine of rabbits injected with  $\alpha$ -picoline. Thus rabbits, lacking the ability to methyrate pyridine compounds, are able to oxidize  $\alpha$ -picoline to picolinic acid. The conjugation of picolinic acid with glycine is analogous to the synthesis of hippuric acid from benzoic acid and glycine, a well known detoxication process in many vertebrates. In 1912 Ackermann (20) presented evidence that a dog fed large amounts of nicotinic acid (9 gm. of nicotinic acid as sodium nicotinate over a 5 day period) excreted about equal amounts of trigonelline and nicotinuric acid, the dipeptide of nicotinic acid and glycine. No change in the condition of the dog was observed during the administration of these large amounts of nicotinic acid along with a normal ration. Ackermann performed the experiment with only one dog which he described as middle-sized. Upon duplication of Ackermann's work in our laboratory<sup>4</sup> with a small dog (4.6 kilos) definite toxicity was observed after 5 days of nicotinic acid administration (2 gm. per day). The dog failed to eat and showed spasmodic vomiting. Upon discontinuing the nicotinic acid, the dog returned to normal.

In 1926 Tomita, Komori, and Sendju (21) fed nicotinuric acid prepared according to the method of Ackermann to polyneuritic pigeons without effect. However, they reported that 0.5 gm. of sodium nicotinate by injection, or 1 gm. by stomach tube, proved toxic to pigeons. Thus the ability of dogs to handle large amounts of nicotinic acid is undoubtedly dependent upon their ability to conjugate at least part of that absorbed with glycine. Animals that are not able to bring about this conjugation so readily will probably show greater toxicity to nicotinic acid. However, since humans are undoubtedly similar to dogs in this respect, no difficulty should be encountered from the use of reasonable amounts of nicotinic acid in humans.

<sup>4</sup> We are indebted to D. V. Frost for the availability of these data.

# SUMMARY

1. The factor necessary for the cure and prevention of black tongue in dogs produced on a modified Goldberger diet has been isolated from liver and identified as nicotinic acid amide.

2. Both nicotinic acid and nicotinic acid amide are effective in curing black tongue in dogs and in maintaining dogs in a normal condition on the basal black tongue-producing diet.

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## TRYPTOPHANE METABOLISM

### IX. THE EXCRETION OF KYNURENIC ACID IN THE BILE AND URINE OF THE DOG AFTER THE ADMINISTRATION OF KYNURENIC ACID AND *l*- AND *dl*-TRYPTOPHANE\*

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In all of the early studies of kynurenic acid excretion in the dog only the urine was analyzed. A few years ago Kotake and Ichihara (1927, 1931) noted that the bile of this species may contain kynurenic acid in variable amounts. Their experimental evidence was confined to four trials with tryptophane and two with kynurenic acid, both injected subcutaneously. Their observation that kynurenic acid is excreted in the bile of the dog has not had further confirmation so far as we are aware; we therefore undertook the more comprehensive study summarized in this paper.

Our data on the feeding of tryptophane and on the subcutaneous administration of kynurenic acid confirm in general the observations of Kotake and Ichihara. However, attempts to recover kynurenic acid given by mouth have yielded results which lead us to question their assumption that the irregular and incomplete urinary recoveries of kynurenic acid which they have tabulated from the literature are due primarily to diverted excretion of kynurenic acid in the bile.

\* The experimental data are taken from a dissertation submitted by John T. Correll in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa. For some of the earlier work on this problem we are indebted to Dr. Lyle C. Bauguess.

A preliminary report of this work was presented before the American Society of Biological Chemists at Detroit, April, 1935 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 109, p. xxiv (1935)).

A portion of this paper deals also with kynurenic acid excretion after the feeding of *dl*-tryptophane, which has been administered to rabbits (Matsuoka, Takemura, and Yoshimatsu, 1925; Berg, 1934) but not to dogs. Our chief purpose in using *dl*-tryptophane was to make sure that an unexpected difference did not exist between its use for kynurenic acid production in the rabbit and in the dog; the *d* form is used for growth by the rat (Berg, 1934) but not by the mouse (Kotake, Ichihara, and Nakata, 1936).

#### EXPERIMENTAL

For these studies bile fistula dogs were prepared by the Rous and McMaster (1923) technique. The cannula used was of German silver, instead of glass, to provide a thinner wall and larger opening, thus lessening the chances of constriction. The rubber (stethoscope) tubing was supported by enclosure in a fold of the mesentery; such an arrangement may also guard against possible access of organisms along the course of the tubing. A para rubber glove served admirably as the collecting bag; the reed basket was covered with adhesive tape to prevent the stems from wearing loose and puncturing the glove; the canvas jacket was provided with holes for the front legs. Sterile precautions were observed throughout the study. The collecting tube was closed with a Hoffmann screw clamp between collections; the exterior end was filled with a 4 per cent phenol solution immediately after each collection and covered with a sterile sponge saturated with the same antiseptic.

Female dogs were used to facilitate catheterization. During experimental periods the bile and urine were collected once each day at the same hour. Occasionally the bile was cultured, but no indication of infection was found. According to Smith, Groth, and Whipple (1928), infection with common air-borne spore bearers (*Bacillus subtilis*, for example) causes no clinical disturbance and produces no change in bile salt metabolism. It seems unlikely that it should affect kynurenic acid content. Occasional fluctuations noted in the volume of bile were probably due to temporary obstructions, such as small kinks in the bile duct at the point of insertion of the cannula or plugs of mucus or precipitate along the course of the tubing.

The dogs were housed in metabolism cages and were fed a

weighed basal diet. In some of the experiments the standard salmon-bread diet employed by Smith, Groth, and Whipple (1928) was used; in others, commercial Purina Dog Chow was fed. The food was given as a mash. Tryptophane or kynurenic acid supplements, when fed, were mixed intimately with a small portion of the normal daily ration and offered first; the rest of the diet was fed after this had been consumed. On a few occasions the supplement had to be fed by hand.

The *l*-tryptophane was prepared as directed by Cox and King (1930). The *dl*-tryptophane was made from the former essentially as directed by Matsuoka, Takemura, and Yoshimatsu (1925). The purity of these products was checked by melting point determinations and nitrogen and optical analyses. Part of the kynurenic acid employed was prepared by partially purifying urinary kynurenic acid precipitates from dogs or rabbits, as indicated by Berg (1931). A synthetic product (Hoffmann-La Roche, Inc.) was also used. The identity of the two was established by individual and mixed melting points and by response to the Jaffe (1882-83) test.

Both bile and urine were analyzed for kynurenic acid daily by the Capaldi (1897) procedure, essentially as previously outlined (Berg, 1931). The bile precipitates were always contaminated with a viscid, mucin-like substance that made filtration extremely slow. Attempts to remedy this difficulty by first hydrolyzing the sample with 4 per cent hydrochloric acid, 15 per cent hydrochloric acid, or 10 per cent sodium hydroxide on a steam bath for 18 to 24 hours were unsuccessful. Therefore, to avoid the necessity of filtering and permit separating and washing by centrifugation, the precipitations from bile were carried out in 50 cc. centrifuge tubes. All final precipitates were washed with water-saturated *n*-butyl alcohol to remove possible contaminants (Berg, 1931).

Four trials of the modified procedure for bile were made on aliquots of bile to which 0.2 gm. of kynurenic acid had been added. In each case the apparent kynurenic acid content of an equal aliquot was determined and subtracted. The net recoveries of kynurenic acid varied from 0.1869 to 0.1940 gm. or from 93.4 to 97.0 per cent. In tests on urine net recoveries of 0.5 to 1.0 gm. of added kynurenic acid ranged from 88.0 to 93.0 per cent. In one instance in which breakage prevented making the final butyl alcohol washing the recovery was 105 per cent.

## DISCUSSION

The results of a series of studies on the subcutaneous and oral administration of kynurenic acid to bile fistula dogs are given in Table I. When kynurenic acid was injected, the total recoveries

TABLE I

*Recoveries of Kynurenic Acid from Urine and Bile after Its Administration to Dogs*

Dog No.	Average weight during test	Kynurenic acid administered	Net* kynurenic acid recovered			
			From bile		From urine	
	kg.	gm.	gm.	per cent	gm.	per cent
1	8.4	0.5 (Subcutaneously)	0.0013	0.3	0.4172	83.4
		0.5 "	0.0719	14.4	0.3983	79.7
		0.5 "	0.1648	33.0	0.3109	62.2
		0.5 "	0.0904	18.1	0.3824	76.5
6	7.7	0.5 "	0.0036	0.7	0.4114	82.3
		0.5 "	0.0009	0.2	0.3342	66.8
		0.5 "	0.0000	0.0	0.3484	69.7
		1.5 "	0.0192	1.3	0.9451	63.0
7†	10.8	1.0 "			0.7342	73.4
		1.0 "			0.7212	72.1
		1.0 "			0.7517	75.2
3	7.2	2.0 (Per os)	0.0287	1.4	0.7547	37.7
5	10.3	2.0 " "	0.0101	0.5	0.3558	17.8
		2.0 " "	0.0407	2.0	0.2714	13.6
		2.0 " "	0.0000	0.0	0.3162	15.8
		2.0 " "	0.0000	0.0	0.3162	15.8
	10.2	1.0 " "	0.0181	1.8	0.2543	25.4
		1.0 " "	0.0000	0.0	0.2518	25.2
		1.0 " "	0.0102	1.0	0.2365	23.7
		1.0 " "	0.0000	0.0	0.2276	15.1
6‡	8.2	1.5 " "	0.0306	2.0	0.2083	13.9
		1.5 " "	0.0451	3.0	0.2975	19.8
		1.5 " "				

\* The average apparent kynurenic acid isolated on the control days before and after the experimental period has been subtracted from the apparent kynurenic acid found in the 24 hour samples following kynurenic acid administration.

† Dog 7 did not have a bile fistula.

‡ During this 3 day period 0.6620 gm. of kynurenic acid, or 14.7 per cent of the total 4.5 gm. fed, was recovered from the feces.

varied somewhat but were all fairly high (64.3 to 95.2 per cent). However, when kynurenic acid was fed, low total recoveries (15.6

to 39.1 per cent) were obtained. In the literature relatively few studies can be found on subcutaneous or oral administration of kynurenic acid itself. In those which are available, recoveries after feeding were almost always lower than after injection. Solomin (1897) found that isolation from the urine following administration to dogs and rabbits by mouth was always unsatisfactory (22.1 per cent in one instance; 9.4 to 9.6 per cent in three others); after subcutaneous injection, recoveries were much higher (58 and 61 per cent in the rabbit to 83, 88, and 98 per cent in the dog). Hauser (1895) obtained low urinary recoveries in the dog after feeding (36 and 54 per cent). Homer (1915) made only one test by each mode of administration to the dog; she reported 91 per cent recovery from the urine after subcutaneous injection and 81 per cent after feeding. Matsuoka (1917, 1918) isolated 11.9 per cent of the kynurenic acid given to rabbits by mouth, and 71.9 to 98.1 per cent of that introduced subcutaneously. Kotake and Ichihara (1931) report studies on two bile fistula dogs in which kynurenic acid was recovered from both the bile and the urine after its subcutaneous administration; that found in the urine represented 78 and 75.8 per cent of the kynurenic acid injected; that from the bile, 15.2 and 18.6 per cent. They did not make similar tests after administering kynurenic acid by mouth. In the five separate feeding trials on dogs reviewed above the urinary recoveries averaged only 38 per cent as compared with 84 per cent for the six injection tests. The differences which we have found are of the same order.

The data in Table I pertaining to oral administration of kynurenic acid show that the amount found in the bile was far from adequate to account for the discrepancy between the urinary recoveries after feeding and those after subcutaneous injection. One is led to suspect that some of it may not have been absorbed. Solomin (1897) was able to recover 15 per cent or more from the feces of a human subject after feeding kynurenic acid and 10 per cent in a similar trial on the dog. Hauser (1895) failed to find any in the feces of man and only a trace in the feces of the dog. In our studies only one attempt was made to recover kynurenic acid from the feces. After correction for the small amount of material which could be isolated from an aliquot of the same weight of feces from the same dog on the same basal diet, careful

analysis accounted for 14.7 per cent of the kynurenic acid fed. Attempts to recover kynurenic acid mixed with feces and subjected to the same analytical procedure yielded only 30 per cent or less; much of it was very probably either adsorbed by the feces or destroyed by bacterial degradation. We suspect the former. In any event, the 14.7 per cent which was recovered after feeding may be regarded as low, some very probably having escaped isolation or having undergone decomposition in the alimentary tract. This assumption seems reasonable, since from both bile and urine only 17.5 per cent of the amount fed could be recovered.

The product isolated from the bile in these and the following studies on tryptophane has been established as kynurenic acid by the characteristically high melting point, by mixed melting point with kynurenic acid of known purity, and by the Jaffe (1882-83) test.

We have repeatedly verified the observation of Kotake and Ichihara that kynurenic acid is excreted in the bile after *l*-tryptophane administration. Space does not permit including all of the individual tests on tryptophane, or the analyses made each day during the interim periods. We are therefore presenting only the averages of several series (see Table II). Sometimes we had difficulty in inducing our dogs to eat with regularity. Data for such periods have been omitted because of the possibility that changes in regimen, particularly resumption of food consumption after fasting, might affect kynurenic acid output (Matsuoka, 1918). Table II conveys clearly the essential observation that kynurenic acid appeared in the bile in varying amounts every time tryptophane was fed, although the distribution between bile and urine varied considerably. For a given dog over comparable dietary periods and on the same intake of *l*-tryptophane the total kynurenic acid output per day was much less variable than the distribution between bile and urine. This substantiates the observations of Kotake and Ichihara. It is realized that averages mask extreme variations. The figures in Table II give a conservative idea of results usually obtained. In none of approximately 60 individual tests on tryptophane administration did we find a greater net output of kynurenic acid in the bile than in the urine. The greatest proportion ever found in the bile was 48.4 per cent of the total net output; on the other hand, in only one or two of

the tests on the bile was there any doubt that at least a trace of kynurenic acid was present.

In Table II we have included particularly the data which afford comparison between the quantities of kynurenic acid formed from

TABLE II  
*Average Net\* Daily Output of Kynurenic Acid in Bile and Urine after Feeding l- and dl-Tryptophane*

Dog No.	Average weight	Days of feeding†	Tryptophane fed	Kynurenic acid excreted		
				In bile	In urine	Total, per cent of theoretical‡
	kg.		gm. per day	gm. per day	gm. per day	
1	9.5	5- 8	2.0 (l-)	0.0760	0.3612	24.2
	8.3	24-27	5.0 (l-)	0.1236	0.7875	19.7
4	15.8	4- 8	2.5 (l-)	0.2836	0.4419	31.3
	15.6	14-16	5.0 (l-)	0.1139	1.2291	29.0
	14.6	59-60	5.0 (l-)	0.0251	1.5851	34.7
	13.7	65-66	2.5 (l-)	0.0190	0.6556	29.1
	13.7	68-69	10.0 (dl-)	0.0792	1.6508	18.6
	13.6	73-74	5.0 (dl-)	0.0209§	0.8428	18.6
	8.9	3- 4	5.0 (l-)	0.2667	0.9447	26.2
6	8.5	11-13	10.0 (dl-)	0.1013	1.5023	17.3
	8.3	18-20	5.0 (dl-)	0.1177	0.7582	18.9
	7.7	46-48	5.0 (l-)	0.0504	0.9539	21.7

\* The average apparent kynurenic acid isolated on the control days before and after the tryptophane feeding period has been subtracted.

† Dog 1 ate 200 gm. of salmon-bread per day during each period and Dog 4, 300 gm. of Purina Dog Chow. Dog 6 consumed 100 gm. of Purina Dog Chow per day during Days 3 to 4, 125 gm. per day during Days 11 to 13, and 150 gm. per day during the other periods.

‡ Of the total kynurenic acid theoretically synthesizable from l-tryptophane, usually less than 35 per cent is produced. All percentages are based on kynurenic acid equivalent to the total l- or total dl-tryptophane fed.

§ On the 2nd day of this period the bile volume was slightly over half that of the 1st day. The tube was permanently obstructed. Hence this average must be low.

l- and from dl-tryptophane. The dl-tryptophane data also show the irregular distribution of kynurenic acid between bile and urine. In each instance, however, the total output was appreciably less than that after the ingestion of an equal amount of l-tryptophane.



It seems possible that the dog, like the rabbit, does not produce very much kynurenic acid from *d*-tryptophane, probably because as in the rabbit *d*-tryptophane cannot be converted directly into kynurenic acid (Berg, 1934).

On the whole the tendency seemed to be toward the excretion of a somewhat greater proportion of kynurenic acid in the bile after feeding tryptophane than after the administration of kynurenic acid. From 2 to 39 per cent of the kynurenic acid excretion shown in Table II was by way of the bile; the average was 9.1 per cent. The output by the same route in Table I ranged from 0 to 33 per cent, but averaged 5.7 per cent. If this small difference is significant, it may possibly be explained on the basis of a kynurenic acid distribution more favorable to biliary elimination. The studies of Abderhalden, London, and Pincussohn (1909), Matsuoka and Takemura (1922), and Ichihara, Otani, and Tsujimoto (1931) suggest that kynurenic acid is synthesized in the liver. If this is true, a concentration of kynurenic acid may occur at the site of its synthesis within the liver cells and this may facilitate its escape into the bile.

#### SUMMARY

The observation that kynurenic acid is excreted in the bile as well as in the urine of dogs after tryptophane administration has been confirmed.

When kynurenic acid is given by mouth, an appreciable amount apparently escapes absorption; this circumstance, more often than excretion by the bile, accounts for the low kynurenic acid output in the urine after such administration.

*dl*-Tryptophane produces much less kynurenic acid than *l*-tryptophane, probably because *d*-tryptophane does not undergo direct conversion to kynurenic acid in the dog.

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# THE IDENTIFICATION AND QUANTITATIVE DETERMINATION OF VOLATILE ALCOHOLS AND ACIDS\*

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In a recent paper by Friedemann and Klaas (1) a rapid, simple micromethod, applicable to biological materials and highly specific for alcohols as a group, was described. It was pointed out that before the method could be applied for the determination of alcohol it must be shown that only one alcohol is present. Since ethyl alcohol is the only volatile alcohol found in a very large number of biochemical systems (and since in most instances it is the only volatile alcohol present in alcoholic beverages), the substance determined by this method with a high degree of probability is ethyl alcohol. In the presence of two or more alcohols all present micromethods fail. Necessary then is a method for identifying as well as quantitatively determining each alcohol. In biochemical and medicolegal work the sample available for analysis is often small. The method therefore should be sensitive enough to apply to such small samples.

The procedure here described consists of distillation of the sample from acid  $\text{Na}_2\text{WO}_4\text{-HgSO}_4$ , redistillation from  $\text{Ca(OH)}_2\text{-HgO}$ , oxidation of the alcohol by chromic acid to the respective acid, and finally, determination of the distribution constant with ethyl ether. A distribution constant below that of acetic acid indicates the presence of another alcohol or a mixture of alcohols. In such a case the final oxidation mixture from another sample is fractionally distilled; the total acidity and the distribution

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constant are determined in each of the serial distillates. Two physical constants, the rate of distillation and the distribution constant, thus determine the identity of the acids which in turn identifies the alcohols.

Since success of the procedure depends upon the determination of minute quantities of volatile acids, the determination of the latter in various biological materials is also described. The most practical method for the preliminary separation of volatile acids from the sample appears to be steam distillation with acid  $\text{Na}_2\text{WO}_4\text{-MgSO}_4$ . An apparatus for micro steam distillation is described. Formic, pyruvic, crotonic, and other acids, such as lactic acid, are removed by redistillation from acid  $\text{MgSO}_4\text{-HgO}$ . Aeration with  $\text{CO}_2$ -free air at room temperature just before titration with 0.01 *N* NaOH quantitatively removes  $\text{CO}_2$  without loss of volatile acid. The end-point is sharp and permanent, with a maximum error of  $\pm 0.05$  cc.

The distribution principle was first used by Behrens (2) in 1926 for the determination of mixtures of fatty acids. The technique was improved by Werkman (3) and applied to the determination of alcohols by Werkman and Osburn (4). Although excellent for large quantities of alcohols and acids, the procedures are not suitable for minute amounts such as must be determined when the sample available is small. In this paper the writer proposes the following changes in technique. For determination of volatile acids, (1) steam distillation in the presence of salt, instead of direct distillation which requires subsequent additions of water until the distillation is complete; the steam distillation is complete in 15 to 20 minutes; (2) redistillation (instead of refluxing) from acid  $\text{MgSO}_4\text{-HgO}$ , removing interfering acids which can be determined by more specific methods; (3) a short aeration of the sample with  $\text{CO}_2$ -free air, instead of refluxing; (4) simplification of the technique of determination of the distribution constant; (5) in unknown mixtures the acids are identified by their rate of distillation (Duclaux) and the distribution constants of the serial distillates. For determination of alcohols, (1) direct distillation of the sample from inorganic precipitants which prevent foaming and which remove some of the interfering volatile substances; (2) redistillation (refluxing is unnecessary) from  $\text{Ca(OH)}_2\text{-HgO}$  which removes traces of acids, ketones, alde-

hydes, and phenols; (3) oxidation by 2 N  $\text{H}_2\text{SO}_4$ - $\text{K}_2\text{Cr}_2\text{O}_7$  in the receiving flask, a glass-stoppered volumetric flask, at room temperature or on a water bath, instead of refluxing.

### *Description of Methods*

*Apparatus*—The apparatus for steam distillation is shown in Fig. 1. Steam is generated in a 1 gallon galvanized iron kerosene can. Brass tubes, A and B, are inserted and soldered into the

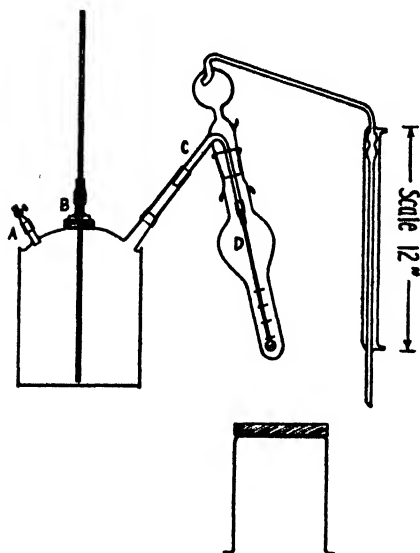


FIG. 1

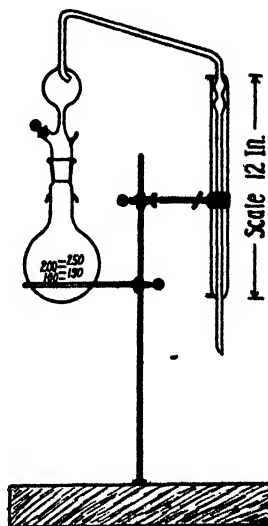


FIG. 2

FIG. 1. Apparatus for steam distillation of volatile acids

FIG. 2. Apparatus for distillation of alcohols and volatile acids

top of the can as shown. The spout of the generator is connected with the glass apparatus by means of a rubber tube of large bore. The inlet tube C should also be large (10 mm. inside diameter) to allow condensed moisture to run back into the generator. The distilling flask is made from a 300 cc. Kjeldahl flask. A No. 20 Pyrex interchangeable ground glass connection is sealed to the bottom. The flask is then sealed off at the top. Calibration marks are made at 10 cc. intervals. If more than one unit is used, it is advisable to make all of the flasks as nearly as possible

of the same length. All inlet tubes, *D*, should also be of the same length. The steam generator is heated by means of a Bunsen burner; the flask by means of a microburner.

Fig. 2 shows the apparatus used in all other distillations. The side arm with a ground glass stopper is necessary for the determination of formic acid. The distilling flask is made from a 500 cc. round bottom flask provided with a No. 20 Pyrex interchangeable ground glass connection. The flask is calibrated at 100, 150, 200, and 250 cc.

Carbon dioxide-free air is obtained from a tower (a glass tube  $3 \times 48$  inches) filled with soda lime. The upper end of the tower is packed with cotton to prevent escape of any particles of soda lime.

#### *Reagents—*

Distilled water may at times contain considerable quantities of volatile material (alcohols, aldehydes, phenols, etc.). This is particularly true of condensed power-house steam and distilled water produced from water contaminated with industrial wastes. The water should be tested before each run. If necessary, it should be redistilled from alkaline  $\text{KMnO}_4$ . It should be stored in a large glass bottle and delivered therefrom through a glass siphon with a *minimum of rubber connections*. The first portion of water from the siphon should be discarded, since it may contain oxidizable impurities from the rubber tubing. Water from a wash bottle should never be used.

Sodium tungstate. A 10 per cent solution.

Sulfuric acid, 10 *N* and 20 *N*.

Magnesium sulfate. Epsom salt, U.S.P.

$\text{HgO}$ . The red oxide.

Mercuric sulfate. 100 gm. of  $\text{HgSO}_4$  are dissolved in 1 liter of 2 *N*  $\text{H}_2\text{SO}_4$ . To prevent precipitation of basic mercuric salt, the  $\text{HgSO}_4$  should be added to 500 cc. of water to which have been added 56 cc. of concentrated  $\text{H}_2\text{SO}_4$ . After heating until solution is complete, the volume is brought to about 1000 cc.

$\text{Ca(OH)}_2$  suspension. 100 gm. of  $\text{CaO}$  (reagent quality) are slaked with a minimum of water. To this is added 1 liter of water. The resulting creamy suspension is kept in a glass-stoppered bottle protected from dust.

Anhydrous ether. This is prepared from U.S.P. ether, or is

reclaimed according to the directions given by Werkman and Osburn. It is allowed to stand for some time over anhydrous  $\text{CaCl}_2$ . A small quantity of soda lime or powdered  $\text{CaO}$  should also be added to neutralize any acids which may be present. The ether is then distilled, preferably from an all-glass still in the presence of fresh  $\text{CaCl}_2$  and lime or soda lime. It is kept in the refrigerator; only slightly more than is to be used in the analyses is removed from the refrigerator and warmed to approximately  $25^\circ$ .

**Standard NaOH.** 0.1 N NaOH is prepared from clear saturated NaOH solution and boiled distilled water. 0.01 N solution is prepared at frequent intervals by dilution with boiled distilled water. These solutions are kept in suitable reservoirs and burettes, guarded against the entrance of  $\text{CO}_2$  by soda lime tubes.

Oxidizing agent for direct determination of ethyl alcohol. See Friedemann and Klaas (1).

*Distillation of Volatile Acids from Biological Materials—Tissues* are frozen and crushed according to the procedure of Graeser, Ginsberg, and Friedemann (5). About 10 gm. of the crushed tissue are transferred to a tared flask (see Fig. 1) which contains 10 cc. of water and 4 cc. of 10 N  $\text{H}_2\text{SO}_4$ .<sup>1</sup> The flask is again weighed.

10 cc. of *blood* are run into the acid mixture, as in the case of tissues. 10 to 25 cc. of *urine*, or 5 to 25 cc. of *culture medium*, are run into 4 cc. of 10 N  $\text{H}_2\text{SO}_4$  plus enough water to bring the volume, with the sample, to 25 to 30 cc. 15 cc. of tungstate are then added. The contents are then mixed thoroughly by rotation of the flask. 8 gm. of  $\text{MgSO}_4$  (6) are added. The contents

<sup>1</sup> The order of addition of acid and tungstate is important; the acid should be added first. The presence of alkali, especially when the sample is heated before acidification, may greatly increase the quantity of volatile acids. The increase is of about the same order as that obtained from blanks which contain approximately the same quantity of sugar as the sample. Since most biochemical materials contain sugar, the determination of total volatile acids after saponification by alkali may yield erroneous results. It should be noted that Werkman and Osburn recommend the distillation of alcohol from the neutralized sample, followed by acidification and distillation of the volatile acids from the residue. While this gives excellent results with very large quantities of alcohol and acids, the writer urges that the residue from the determination of alcohol should not be used for the determination of volatile acids in urine, tissues, etc., which contain relatively very small quantities of alcohol and volatile acids.



are again mixed by rotation, after which the flask is connected to the apparatus and the ground glass connection is wetted.

In the case of blood and tissues, success of the method depends upon the technique of precipitation.<sup>1</sup> The flask is tilted until the contents enter the enlarged upper part. 5 cc. of tungstate are then slowly added, with rotation of the flask in the horizontal position and with frequent mixing with the contents which still remain in the lower narrow part. The latter is accomplished by momentarily bringing the flask to the vertical position. Two more additions of 5 cc. each of tungstate are made, with mixing as before.

The water (distilled water) in the steam generator is heated to boiling. The clamp at A (Fig. 1) is closed and the contents of the flask are rapidly brought to boiling. If the mixture foams, clamp A should be opened and the microburner should be removed. In most instances (unless an insufficient amount of tungstate is present) no foaming is encountered when the distillation is again resumed. The flame under the steam generator should be low, but the microburner should be high enough to reduce rapidly the volume to 30 cc. The Bunsen burner flame is then increased. The microburner flame is adjusted to maintain the volume at approximately 30 cc. 200 cc. of distillate are collected in the 500 cc. round bottom flask shown in Fig. 2. From 15 to 20 minutes are required for the steam distillation.

*Redistillation of Volatile Acids*—From 1 to 5 per cent of lactic acid and from 5 to 20 per cent of pyruvic acid come over in the steam distillate. For this reason it is necessary to redistil the distillate before carrying out any titrations. Since the presence of formic acid complicates the calculations by introducing an additional variable and since it can be determined by more specific methods, it is desirable to remove this acid also.<sup>2</sup> This is accomplished by simply distilling with  $\text{HgO}$  (3) or  $\text{HgSO}_4$  in acid solution. Distillation with mercuric salts and  $\text{MgSO}_4$  also re-

<sup>1</sup> The distribution constant of formic acid is 91.0, only 4.5 greater than for acetic acid (see Fig. 3). The presence of relatively large quantities of formic acid in a solution of acetic and formic acids thus increases the constant but slightly and therefore introduces a large error in the determination of acetic acid.

moves pyruvic acid quantitatively and about 75 per cent of crotonic acid.<sup>3</sup>

2 cc. of 10 N  $\text{H}_2\text{SO}_4$ , 50 gm. of  $\text{MgSO}_4$  (measured with a 50 cc. beaker), and 0.2 to 0.3 gm. of  $\text{HgO}$  (measured by a small spoon) are added in the order indicated. If  $\text{HgSO}_4$  is to be used, add the  $\text{MgSO}_4$ , 10 cc. of  $\text{HgSO}_4$  in 2 N  $\text{H}_2\text{SO}_4$  (see "Reagents"), and talcum. Excessive foaming is encountered often when larger quantities of acid or  $\text{HgO}$  have been added. The flask is connected to the apparatus and the ground glass connection is wetted. The contents are rapidly brought to boiling. The results are more uniform if, at this point, the flame is removed for a short time. The distillation is now resumed and carried out at the rate of 4 to 5 cc. of distillate per minute. The *flask should be carefully watched* when the volume becomes less than 100 cc.; *the distillation should be discontinued as soon as the contents begin to crystallize*. The redistillation requires from 30 to 45 minutes.

If the lactic, formic, or crotonic acid content is relatively very great, it may be necessary to redistil a second time from acid  $\text{MgSO}_4\text{-HgO}$ . In most instances, however, one redistillation appears to be sufficient.<sup>4</sup> The final distillate is collected either in a 250 cc. volumetric flask or in a 500 cc.-Erlenmeyer flask, depending upon the quantity of acid present or whether the distribution constant is to be determined.

Blank distillations should be made with the same water and reagents.

*Modified Duclaux Distillation*—The solution or distillate in the 500 cc. round bottom flask is brought to a volume of 200 cc. 2 cc. of 10 N  $\text{H}_2\text{SO}_4$ , 50 gm. of  $\text{MgSO}_4$ , and 0.2 to 0.3 gm. of  $\text{HgO}$

<sup>3</sup> Refluxing (Werkman) with acid  $\text{HgO}$  to remove formic acid, etc., is unnecessary with the small quantities of total acid (1 to 100 cc. of 0.01 N) present in the aliquots recommended.

<sup>4</sup> A second redistillation is apparently unnecessary in the case of normal tissues and urine. However, if a large volume of urine is distilled with steam, considerable quantities of volatile acids, which require at least two redistillations for removal, are found in the steam distillate. During the long period of steam distillation, from 2 to 4 hours required for the large sample, no doubt considerable decomposition of labile substances and volatilization of relatively non-volatile acids occur.

are added and the distillation is carried out as described above. The distillate is collected in large test-tubes (25 × 250 mm.) in fractions as follows: 25, 25, 25, 25, 50 cc., and to crystallization (about 40 cc.). The tubes are calibrated at 25, 50, and 52 cc. The volume is brought to the 52 cc. mark. A 25 cc. aliquot is

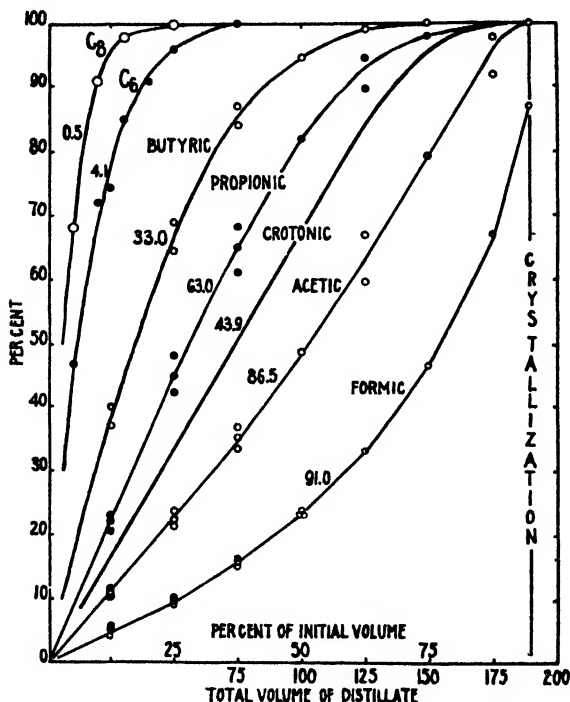


FIG. 3. Rate of distillation of volatile acids. The initial volume of the solutions was 200 cc. To this were added 2 cc. of 10 N  $H_2SO_4$ , 50 gm. of  $MgSO_4 \cdot 7H_2O$ , and 0.2 to 0.3 gm. of  $HgO$ . The distillation required from 30 to 45 minutes.

then diluted, aerated, and titrated as described below. The other 25 cc. aliquot may be used for determination of the distribution constant (see below).

The rate of distillation under the conditions described, without particular care as to size of flame during the distillation, accurate weight of the salt, or accurate adjustment of the initial volume, can be seen in Fig. 3. The approximate acid content of each of

the fractions is shown in Table I. Even under these conditions the results are fairly uniform.

The volatility of formic acid is only slightly increased by the presence of the salt, in confirmation of the work of Witzemann (7). However, the volatility of the other acids is greatly increased. Thus the first 100 cc. of distillate from  $\text{MgSO}_4$  contain approximately 11 per cent more of the acetic acid, 14 per cent more of the propionic acid, and 19 per cent more of the butyric acid. The salt thus allows a better separation of acetic from the higher fatty acids. With  $\text{MgSO}_4$  present it is possible to distil about 99 per cent of the acetic acid. In the absence of  $\text{MgSO}_4$  it is difficult to distil over all of the acetic acid; the total acetic acid yield is therefore less uniform.  $\text{MgSO}_4$  is preferred over

TABLE I  
*Approximate Per Cent of Acid in Serial Distillates*

Acid	1st fraction, 25 cc.	2nd fraction, 25 cc.	3rd fraction, 25 cc.	4th fraction, 25 cc.	5th fraction, 50 cc.	6th fraction, ± 40 cc.
Acetic.....	11	12	12	14	27	24
Propionic.....	23	22	19	17	16	3
Butyric.....	39	30	19	10	2	
Caproic.....	73	22	5		-	
Caprylic.....	96	4				

$\text{Na}_2\text{SO}_4$  or  $\text{NaCl}$  because it is more soluble; a greater yield of distillate can be obtained before crystallization. It does not yield a volatile acid, as might be with the case with  $\text{NaCl}$ .

*Titration of Volatile Acids*—All distillates contain variable quantities of  $\text{CO}_2$  which must be removed completely before titration. Removal of  $\text{CO}_2$  is quickly accomplished by rapid aeration with  $\text{CO}_2$ -free air. Thus, only 30 to 45 seconds suffice to free 25 cc. of a saturated aqueous solution of all  $\text{CO}_2$ . In practice, however, it is advisable to aerate longer. The aeration does not remove any measurable quantity of volatile acid.

An aliquot is transferred to an Erlenmeyer flask or a large test-tube. To minimize losses due to aeration, dilution of all aliquots to about 100 cc. (the required volume added from a graduated cylinder) is recommended. The flask is tilted at an angle of about  $45^\circ$  during the aeration. If the total volume is

25 cc., the sample is rapidly aerated 3 minutes; for 100 cc., 5 minutes; 250 cc., 10 minutes. At the end of aeration the air inlet tube is shaken to remove adhering drops; it is not washed with a stream of water, for this would introduce  $\text{CO}_2$ . The flask, or test-tube if the volume is only 25 cc., should then be covered with a beaker and as soon as possible titrated with 0.01 N NaOH. To the volumes of solutions mentioned above add 1, 2, or 3 drops, respectively, of 1 per cent alcoholic solution of phenolphthalein. The titration is continued to the first permanent faintly pink color.

The same volume of the blank should be treated in the same manner and titrated as the unknown.

*Determination of Distribution Constant*—The determination is carried out with long pear-shaped Pyrex separatory funnels of 250 cc. capacity. These should be cleaned with strong cleaning solutions only at infrequent intervals, and, if so cleaned, should be washed very carefully, dried, and the stop-cock should be carefully greased. Hot water usually suffices to remove greasy matter from the walls. Just before use they should be rinsed with distilled water and allowed to drain a few minutes. The stop-cock is then closed. An aliquot, equal to the aliquot titrated directly, is introduced. Enough water to bring the volume to 100 cc. is added from a graduated cylinder. 50.5 cc. of ether are added from a 50 cc. graduated cylinder. The separatory funnel is now closed, the contents are gently rotated, and the stopper is momentarily opened to relieve the pressure. Equilibrium between the aqueous and ether layers is reached with approximately 15 seconds of vigorous shaking. However, to obtain more uniform results the shaking should be continued for at least 1 minute. The funnel is then allowed to stand several minutes. Almost all of the aqueous layer is drawn off into a 300 cc. Erlenmeyer flask. The funnel is closed and given a circular motion to bring down any drops adhering to the walls. The remaining aqueous layer is now removed. The aeration and titration are carried out as already described.

*Calculations*— $A$  = cc. of 0.01 N NaOH, minus the blank, required *before* ether extraction.  $B$  = cc. of 0.01 N NaOH, minus the blank, required *after* the ether extraction. The constant,  $K = B/A \times 100$ .  $K$ , therefore, represents the *percentage of acid*

which remains in the aqueous phase. The constants obtained under these conditions are shown in Fig. 3. As shown by Werkman and Osburn,<sup>5</sup>  $K$  of a mixture of acids is the sum of the percentage distribution constant of each of the acids present. Thus in a mixture consisting of 25.2 cc. of acetic acid, 26.1 cc. of propionic acid, 21.1 cc. of butyric acid, and 3.8 cc. of caproic acid, a total of 76.2 cc. of 0.01  $N$  acid in 250 cc., a 25 cc. aliquot contained 7.78 — 0.12 or 7.66 cc. before ether extraction and 4.67 — 0.12 or 4.55 cc. after ether extraction.  $K = 4.55/7.66 \times 100 = 59.4$ .  $K$ , calculated, is  $28.6 + 21.6 + 9.1 + 0.2 = 59.5$ .

Greater accuracy could, of course, be obtained by previously drying the separatory funnel, by more carefully measuring all solutions, including the ether, and by maintaining the temperature at 25°. Starting with a wet separatory funnel is preferable, for it allows determinations to be made in rapid succession with the same funnel. The small quantity of water adhering to the wall is within the error of measurement of the graduated cylinders. By drawing off and titrating all of the aqueous solution the use of an additional pipette (Werkman and Osburn) is avoided. By measuring the ether from a burette it would be unnecessary to allow approximately 0.5 cc. for evaporation. While no marked differences have been noted at room temperatures, the best results are obtained at 25°, as recommended by Werkman and Osburn. At temperatures below 20° the tendency to form an emulsion is greater, and more time is required for separation of the layers; at temperatures above 25° the evaporation of ether introduces a large error.

Practically the same results are obtained with smaller volumes, when the same aqueous solution-ether ratio, with smaller separatory funnels and with more dilute NaOH, is kept. The constant is practically the same also with larger quantities of acid which require the use of 0.1  $N$  NaOH.

Constants have also been determined with other aqueous solution-ether ratios. *The following constants were obtained with a 1:1 aqueous solution-ether ratio, acetic acid 73.5, propionic acid 42.4, butyric acid 18.2, crotonic acid 25.6, isobutyric acid 16.8.*

<sup>5</sup> For discussions of methods for the calculation of constants and their application to mixtures of fatty acid, the reader is referred to the papers by Werkman (3) and Werkman and Osburn (4).

The differences in the constants for acetic, propionic, and butyric acids are somewhat higher with the 1:1 ratio. However, the difference between the constants for butyric and caproic acids is considerably smaller.

*Formic Acid*—Practically all of the methods for the determination of formic acid depend upon its oxidation by either  $\text{KMnO}_4$  or  $\text{HgCl}_2$ .  $\text{KMnO}_4$  readily oxidizes a great many substances besides formic acid, and for this reason it should not be used on distillates from biological materials. On the other hand  $\text{HgCl}_2$  in slightly acid solution oxidizes relatively few substances. Aldehydes and ketones form insoluble salts, which may introduce an error when the precipitate is weighed; they do not markedly interfere when the extent of the reduction after several hours of heating is determined iodometrically, or when the  $\text{CO}_2$  produced by the oxidation is determined. In the writer's experience the most likely substance to interfere is pyruvic acid, which fortunately is present in very small quantity, if at all, in most materials. With the possible exception of pyruvic acid, therefore, the oxidation of distillates with  $\text{HgCl}_2$  is quite specific for formic acid.

Gravimetric and iodometric methods fail when the quantity of acid is very small. With slight changes, the method of Werkman (3) can be adapted for determination of the  $\text{CO}_2$ . The apparatus and absorption tower recommended are those of Friedemann and Kendall (8). The oxidation is carried out with  $\text{HgCl}_2$  or  $\text{HgSO}_4$ , solutions of which are added through the separatory funnel. The procedure, although highly specific, requires considerable skill and is time-consuming.

The following procedure is simpler, although somewhat less specific. The steam distillate is redistilled with  $\text{H}_2\text{SO}_4$ ,  $\text{MgSO}_4$ , and talcum, but without  $\text{HgO}$ , as already described. This removes lactic acid and most of the pyruvic acid. The distillation is discontinued when the residue becomes syrupy or begins to crystallize. 25 cc. of water are then added through the side tube and the distillation is continued. Two or three more additions of 25 cc. each are made. The distillate is collected in another 500 cc. round bottom distillation flask. It is thoroughly aerated and then titrated with 0.01 N  $\text{NaOH}$  (aqueous sodium phenolphthaleinate is preferred to the alcoholic solution). The residue from the titration is then redistilled from  $\text{H}_2\text{SO}_4$ - $\text{MgSO}_4$ - $\text{HgO}$ , aerated,

and again titrated. The difference between the two titrations (minus blanks) represents formic acid removed by the  $\text{HgO}$ .

*Identification of Volatile Acids in Distillate*—Neither the Duclaux titration nor the distribution constant alone suffices for the identification of individual acids in an unknown mixture, as is illustrated by the following analyses of urine. An acidified 24 hour sample, 2255 cc., was steam-distilled with 100 gm. of  $\text{Na}_2\text{WO}_4$  and 150 cc. of 10 N  $\text{H}_2\text{SO}_4$  in an all-glass apparatus. 6 volumes of distillate were collected. The distillate was made strongly alkaline and was then evaporated to a small volume on the steam bath. The results after two distillations from  $\text{H}_2\text{SO}_4$ - $\text{MgSO}_4$ - $\text{HgO}$  were as follows: total volatile acids, 0.96 cc. N;  $K$ , 75.2. The constant indicates the presence of acetic acid, since the determined  $K$  is greater than that of other fatty acids, but it gives no clue as to the identity of the other acids. Another 24 hour sample, 625 cc., was similarly distilled. The final distillate was collected in five fractions, 25, 25, 25, 25, 50, and  $\pm 40$  cc. These contained 13.5, 12.8, 12.9, 13.7, 32.1, and 23.4 cc. of 0.01 N acid respectively, or a total of 1.08 cc. N. The percentages of volatile acid in each of the distillates were 12.5, 11.8, 11.9, 12.6, 29.6, and 21.6. A third 24 hour sample, 955 cc., contained 0.76 cc. of N acid; the percentages of acid in each of four final fractions (50, 50, 50,  $\pm 40$  cc.) were 24.5, 24.3, 28.5, and 22.7. Both of these fractional distillations (Duclaux) agree, within the limits of error, with the results obtained on distillation of pure acetic acid (Table I). It might be concluded, therefore, that these samples of urine contained only acetic acid. However, determinations of  $K$  on the same distillates indicate the presence of other acids. Thus the values for  $K$  of the six fractions of Sample 2 were 67.1, 77.0, 79.4, 78.5, 76.4, 72.1. For Sample 3,  $K$  for each of the four fractions was 70.9, 81.3, 82.4, 78.4. These determinations of  $K$ , together with the Duclaux titrations, unquestionably point to the presence of acetic acid in urine. Of the other acids, some are apparently slightly more volatile than acetic acid and others less volatile; and their constants are considerably lower than that of acetic acid, as can be inferred from the fact that  $K$  rapidly rises to a maximum and then diminishes. These analyses are cited to emphasize the point that *the determination of  $K$  of serial distillates and the Duclaux titration together are a valuable*



*aid in the identification of individual acids.* Even in the case of urine it was possible thus to identify acetic acid with a fair degree of certainty and to obtain a clue as to some of the properties of the other volatile acids.

Fortunately most biochemical materials contain only a few volatile acids. Thus, the commoner pathogenic bacteria produce only acetic acid (Table IV), which is identified by the exact agreement of the Duclaux titration with that for pure acetic

TABLE II

*Rate of Distillation and Distribution Constants of Serial Distillates*

Volatile acids, cc. of 0.01 N equivalents: acetic 25.2, propionic 26.1, butyric 21.1.

Distillation No.	Acids	1st 50 cc.		2nd 50 cc.		3rd 50 cc.		4th fraction, ±40 cc.	
		Per cent of total acid	K	Per cent of total acid	K	Per cent of total acid	K	Per cent of total acid	K
1	Acetic, propionic	34	70.0	27	72.7	25	78.4	14	85
2	“ butyric	42	47.8	27	59.3	19		12	87
3	“ “	42	48.7	28	60.7	19	81.0	11	87
4	“ “ caproic (7.7 cc.)	50	35.6	24	60.8	16	80.7	10	87
5	Propionic, butyric	53	46.2	33	50.2	13	58.3	1	?
6	Acetic, propionic, butyric, caproic (3.8 cc.)	44	47.2	28	60.1	19	71.4	9	83
7	Acetic, propionic, butyric, caproic (3.8 cc.), caprylic (6.0 cc.)	48	40.6	27	60.1	17	72.2	8	85

acid and *K* exactly equal to that for pure acetic acid for *each of the serial distillates*.<sup>6</sup> Meat extract and meat infusion culture media contain principally acetic and butyric acids. Some bacteria produce acetic and propionic acids; others produce acetic and butyric acids. Mixtures of three or more acids are rarely encountered.

The method of identification of acids in a mixture containing only two volatile acids will be briefly described. Examples will be taken from Table II, which shows results of titrations and

<sup>6</sup> After correcting for the acids in the uninoculated culture medium.

determinations of  $K$  of serial distillates. By inspection of the values for  $K$ , it will be noted that the initial  $K$ , in the case of the acetic-propionic acid mixture, is above 63.0, that  $K$  increases slowly, and that it never reaches the value characteristic for acetic acid, 87. In the case of the acetic-butyric acid mixture, the initial  $K$  is low; it increases rapidly in subsequent fractions and in the final fraction attains a value of 87. Results of Distillation 3, made several days later, are included in Table II to indicate the extent of variations in duplicate samples. In the case of a propionic-butyric acid mixture most of the acid is found in the first 100 cc. of the distillate, since both are rapidly volatilized; the last fraction contains practically no acid. The initial  $K$  is below 63.0; it rises slowly, and never goes above 63.0. Caproic and caprylic acids distil over in the first 50 cc. of distillate. All of the results of Table II can be predicted from the data shown in Table I. It is thus possible by inspection of the data to identify the acids fairly accurately. A few simple calculations suffice for a more complete identification.

Although, in the case of Distillation 1, the results by inspection point to the presence of acetic and propionic acids, the possibility of the presence of acetic and butyric acids will nevertheless be considered.  $K$ , 70.0, of the first 50 cc. fraction corresponds to an acetic to butyric acid ratio of 69:31. The calculated acetic acid content is therefore 23.5 per cent of the total acid present in the sample, and the butyric acid content is 10.5 per cent. This fraction therefore contains 34 per cent of the total acid (see Table II). From  $K$ , 72.7, of the second 50 cc. fraction, acetic acid constitutes 20.0 per cent and butyric acid 7.0 per cent of the total acid of the sample. The third fraction contains 21.3 per cent of the acid as acetic and 3.8 per cent as butyric acid. The fourth fraction contains 13.6 per cent of the acid as acetic and only 0.4 per cent as butyric acid. According to the calculations, therefore, 78.3 per cent of the total volatile acid is acetic acid and 21.7 per cent is butyric acid. The calculated rates of distillation of these acids expressed in per cent of the total of each acid are as follows: acetic acid, 30, 26, 27, 17; butyric acid, 49, 32, 18, 2. None of these rates corresponds with the data for acetic and butyric acids shown in Table I. On the other hand, by assuming the presence of acetic and propionic acids, the calculated rates of

distillation are, for acetic acid, 20, 22, 32, 26; for propionic acid, 49, 32, 17, 2. *Both* of these calculated rates agree fairly closely with the rates determined for the pure acids. Similar calculations in the case of Distillation 3, assuming the presence of acetic and butyric acids, yield results as follows: acetic acid, 22, 27, 31, 20; butyric acid, 66, 30, 4, 0. *Both* of these rates agree fairly closely with the rates determined for acetic and butyric acids.

*Quantitative Determination of Two or Three Volatile Acids*—If only two acids are present, the determination of  $K$  and the total acidity of the entire distillate determine accurately the quantity of each present. The relative quantity of the acids can be determined most conveniently from a graph. Thus, in the case of butyric and acetic acids, the curve is determined by a line drawn from the point 33.0, 100 butyric or 0.0 acetic to the point 86.5, 0.0 butyric or 100 acetic. On this curve, for example,  $K = 70.0$  corresponds to a mixture consisting of 69.3 per cent acetic and 30.7 per cent butyric acid.

With samples which contain three volatile acids, two determinations of  $K$  are necessary, as pointed out by Osburn and Werkman. These authors extract one aliquot of the total distillate with ethyl ether and another with isopropyl ether. If, however, it is desired to use only ethyl ether, the following procedure, which has an accuracy of  $\pm 5$  per cent is recommended. Two fractions, one consisting of the first 100 cc. of distillate and the other consisting of the remainder of the distillate, are collected and the volumes are brought to the mark.  $K_1$  and  $K_2$  represent the respective distribution constants of the first and second fractions;  $X_1$  and  $X_2$  represent their respective calculated total acidities. To illustrate the calculations the presence of acetic, propionic, and butyric acids will be assumed. Then  $A$ ,  $P$ , and  $B$  represent the *total* quantity of each present in the two distillates. The rates of distillation of the acids from the mixture are assumed to be the same as shown in Table I.

Then

$$\frac{0.49 \times 86.5A}{X_1} + \frac{0.81 \times 63P}{X_1} + \frac{0.98 \times 33B}{X_1} = K_1$$

$$\frac{0.51 \times 86.5A}{X_2} + \frac{0.19 \times 63P}{X_2} + \frac{0.02 \times 33B}{X_2} = K_2$$

and

$$A + P + B = X_1 + X_2$$

When solved for  $A$ ,  $P$ , and  $B$ ,

$$A = 0.505 (X_1 + X_2) - 0.0162 K_1 X_1 + 0.0268 K_2 X_2$$

$$P = -1.998 (X_1 + X_2) + 0.0622 K_1 X_1 - 0.0145 K_2 X_2$$

$$B = 2.495 (X_1 + X_2) - 0.0460 K_1 X_1 - 0.0123 K_2 X_2$$

In the case of samples which contain acetic, butyric, and other higher volatile fatty acids ( $C_6$ ,  $C_8$ , etc.), the calculations may be made as follows:  $A$  and  $B$ , as before, represent the total quantity of acetic and butyric acids in the two distillates.  $C$  similarly represents all of the higher fatty acids. This is possible in the calculations, since their distribution constants are very small. An average  $K$  of 2.0 may be assumed.

Then

$$\frac{0.49 \times 86.5A}{X_1} + \frac{0.98 \times 33B}{X_1} + \frac{1.00 \times 2C}{X_1} = K_1$$

$$\frac{0.51 \times 86.5A}{X_2} + \frac{0.02 \times 33B}{X_2} = K_2$$

$$A + B + C = X_1 + X_2$$

When solved for  $A$ ,  $B$ , and  $C$ ,

$$A = 0.0017 (X_1 + X_2) - 0.0005 K_1 X_1 + 0.0232 K_2 X_2^*$$

$$B = -0.0674 (X_1 + X_2) + 0.0337 K_1 X_1 - 0.0309 K_2 X_2$$

$$C = 1.065 (X_1 + X_2) - 0.0331 K_1 X_1 + 0.0077 K_2 X_2$$

\* The first two members of the equation yield such small numerical values that they can be disregarded in most computations. Therefore,  $A = 0.0232 K_2 X_2$ .

*Distillation of Alcohols from Biological Materials*—5 to 25 cc. or gm. of the sample are introduced into a 500 cc. round bottom flask (Fig. 2) which contains approximately 100 cc. of  $H_2O$  and 10 cc. of  $HgSO_4$  solution. This is followed by 15 cc. of tungstate,<sup>7</sup> which is added with rotation of the flask. Enough water is added to bring the volume to about 200 cc. The flask is connected to

<sup>7</sup> In the case of blood, larger volumes of the precipitating reagents should be added for samples greater than 5.0 cc.

the apparatus and the ground glass connection is wetted. 100 cc. or more of distillate are collected in another round bottom flask. 5 cc. of  $\text{HgSO}_4$  and an excess of  $\text{Ca(OH)}_2$  are now added, after which the flask is stoppered and vigorously shaken. The stopper is washed with a small amount of water and the volume is brought to about 150 cc. The sample is redistilled into a 100 cc. glass-stoppered volumetric flask. If it is known that only one alcohol is present, the alcohol can be determined directly on an aliquot by the method of Friedemann and Klaas or by any other oxidimetric method.

Distillations should be carried out until one-half or more has been distilled. The first distillation prevents the distillation of volatile amino compounds and a considerable part of the acetone and aldehydes. The second distillation removes most of the phenols, from 96 to 99 per cent of the aldehydes, and almost completely removes acetone. If large quantities of acetone are present, it may be necessary to distil again from alkaline  $\text{HgO}$ . The odoriferous substances of urine, soil, etc., and many unsaturated volatile substances are not removed completely by the reagents of the first two distillations. However, they are effectively removed by distillation with acid  $\text{KMnO}_4\text{-MnSO}_4$  (9). Approximately 10 cc. of a 10 per cent solution of  $\text{MnSO}_4$  in  $\text{N H}_2\text{SO}_4$  for each 100 cc. of distillate, 5 cc. of 0.1  $\text{N KMnO}_4$ , and talcum are added. Distillation without  $\text{MnSO}_4$  results in a considerable loss of alcohol. The maximum of alcohol lost in each distillation is about 1 per cent.

*Oxidation of Alcohols and Determination of Distribution Constants*—To the distillate (about 80 cc.) are added 10 cc. of 20  $\text{N H}_2\text{SO}_4$  and an excess of powdered  $\text{K}_2\text{Cr}_2\text{O}_7$ . The contents are mixed until solution of the  $\text{K}_2\text{Cr}_2\text{O}_7$  is complete. The stem of the flask and the glass stopper are wetted with the solution. It is then either allowed to stand overnight at room temperature or the flask may be warmed in a water bath. The contents are now transferred to a 500 cc. round bottom distilling flask (Fig. 2). The volume is brought to 200 cc.; 50 gm. of  $\text{MgSO}_4$  and talcum are added. The distillation is carried out as in the case of volatile acids already described. The flame should be removed for a short time when the solution comes to boiling. It is then slowly brought to boiling, after which distillation is rapidly carried to

completion. The distillation should be discontinued *at the first sign of crystallization* in the bottom of the flask. The distillate is collected in a 250 cc. volumetric flask.

Titration of the volatile acid and determination of the distribution constant,  $K$ , are made as already described.

Methyl alcohol is oxidized to formic acid. If its presence is suspected, the entire distillate should be aerated and titrated (aqueous sodium phenolphthaleinate is preferred to the alcoholic solution). It should then be redistilled from acid  $\text{MgSO}_4 \cdot \text{HgO}$  to remove the formic acid. The difference in acid content determines the methyl alcohol. The formic acid yield is not quantitative. Isopropyl alcohol is oxidized largely to acetone (4). If its presence is suspected, the distillate is cooled and an aliquot is distilled into cold distilled water. The delivery tube should dip below the surface of the water. Acetone is then determined by the Messinger method as modified by Hubbard (10).

*Ethyl alcohol is oxidized quantitatively to acetic acid;  $K = 86.5$ .* Propyl and butyl alcohols, however, are not quantitatively oxidized to the respective acids. Thus, only 70 per cent of propyl alcohol, oxidized at room temperature, is recovered as propionic acid; 30 per cent is oxidized to acetic acid and  $\text{CO}_2$ ;  $K = 70 \pm 1$ . Under the same conditions, butyl alcohol yields butyric and acetic acids in the same ratio, namely 70 to 30;  $K = 49 \pm 1$ . Werkman and Osburn obtain higher yields by refluxing. The difference is due to some extent to the temperature at which the oxidation is carried out.

*Calculations*—In a sample which contains two alcohols, the relative quantity of each is most conveniently determined from a graph (3, 4). Thus in the case of butyl and ethyl alcohols, the curve is determined by a line drawn from the point 49, 100 butyl or 0.0 ethyl alcohol to the point 86.5, 0.0 butyl or 100 ethyl alcohol.

*Identification of Alcohols*—The original double distillation with  $\text{HgSO}_4$ , the first from acid and the second from alkaline solution, prevents the distillation of volatile acids. It also eliminates many substances which on oxidation with chromic acid yield volatile acids. *A distribution constant of 86 to 87 therefore identifies ethyl alcohol* (see Table III). According to Werkman and Osburn, the oxidation of propyl and butyl alcohols by chromic acid does

not yield formic acid. When such oxidation mixtures are distilled in the usual manner and titrated, redistilled from acid  $\text{MgSO}_4\text{-HgO}$ , and again titrated, no marked differences are noted between the two titrations. The loss on redistillation of acetic, propionic, and butyric acids is rarely greater than 1 per cent. A marked difference points to the presence of formic acid which is derived from methyl alcohol. Since the original double distillation removes preformed acetone almost quantitatively, the presence of

TABLE III  
*Determination of Alcohol in Blood*

Sample No.	Alcohol by Friedemann-Klaas method,* 1 cc. sample	Procedure of present paper,† 5 cc. sample					
		Single distillation from $\text{Na}_2\text{WO}_4\text{-HgSO}_4$			Double distillation: from $\text{Na}_2\text{WO}_4\text{-HgSO}_4$ , redistilled from alkaline $\text{HgO}$		
		Aliquot titration minus blank	Calculated alcohol	K	Aliquot titration minus blank	Calculated alcohol	K
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	<i>mg. per cent</i>	<i>cc.</i>	<i>mg. per cent</i>		<i>cc.</i>	<i>mg. per cent</i>	
1	36	1.73	40	87	1.80	42	90
2	147	6.33	147	87.2	6.54	152	87.5
3	167	7.46	173	87.1	7.36	170	87.2
4	277	11.95	278	87.0	11.81	274	86.9
5	324	13.82	321	87.2	13.98	325	87.1

\* Three 1.0 cc. samples distilled from  $\text{Na}_2\text{WO}_4\text{-HgSO}_4$ . The results represent the averages of single determinations on each of the three distillates.

† 5.0 cc. sample. Total volume of final distillate 250 cc.; 100 cc. aliquot titrated with 0.0101 N NaOH; 100 cc. aliquot extracted with ether before titration.

acetone in the chromic acid mixture identifies isopropyl alcohol.

Distribution constants below 86 point to the presence of other acids. Identification of the acids also identifies the alcohols.

*Quantitative Determination of Alcohol in Blood*—Results of analyses of blood by the procedure described are shown in Table III. Two 5 cc. samples were distilled from  $\text{Na}_2\text{WO}_4\text{-HgSO}_4$ . One of the distillates was immediately oxidized by chromic acid; the other was redistilled from alkaline  $\text{HgO}$ , and then oxidized. The final volume of distillate was 250 cc., of which 100 cc. were titrated

directly with 0.0101 N NaOH and 100 cc. were extracted with 50 cc. of ether before titration. The results of the first titration (Columns 3 and 6) varied from 1.73 to 13.98 cc. The calculated alcohol content of the samples (Columns 4 and 7) varied from 40 to 325 mg. per cent. Results of ether extraction are not shown, but the results of calculation of  $K$  are shown in Columns 5 and 8. Blood was also analyzed by the more rapid and sensitive method of Friedemann and Klaas. Because of the limited quantity of blood available, 1 cc. samples were distilled.

The close agreement of the results in Columns 4 and 7 with the results obtained by the method of Friedemann and Klaas (Column 2) demonstrates the reliability of the procedure. The double distillation (Columns 6 and 7) involves no apparent loss of alcohol (compare with Columns 3 and 4). The distribution constants (which are slightly higher than usual) definitely establish the sole presence of ethyl alcohol in all of the samples. *Thus alcohol is not only identified but is also quantitatively determined with a fair degree of accuracy.* With a 5 cc. sample, the minimum alcohol concentration at which the method is still reliable is about 50 mg. per cent.

*Volatile Acids and Alcohol in Cultures of Pathogenic Bacteria*—The reliability of the procedures for alcohols and volatile acids, as applied to complex culture media, can be seen from the following experiment.

Table IV contains data for the volatile acids and alcohol produced by a representative group of pathogenic microorganisms. This includes organisms of the colon-typhoid group, streptococci, staphylococci, and pneumococci. The organisms were grown on meat infusion to which had been added 1 per cent of peptone, 0.75 per cent of  $\text{Na}_2\text{HPO}_4$ , 1 per cent of glucose, and 2 per cent of serum. The initial reaction was pH 7.6. The cultures were incubated 24 hours at  $37.5^\circ$ , after which they were sterilized by an exactly 0.1 volume of N  $\text{H}_2\text{SO}_4$ . All data shown in Table IV are corrected by a blank; i.e., an inoculated medium which was immediately acidified.

Results of alcohol determination by two methods are shown in Columns 2, 3, and 4 (Table IV). The distribution constant (Column 2) in every instance agreed with the constant of acetic acid. These organisms therefore produced only ethyl alcohol. Results obtained by the Friedemann and Klaas method (Column 4) are



in fair agreement with the results obtained by the procedure of the present paper (Column 3).

The volatile acids were determined by two methods (Columns 5 to 8), (a) by the procedure of the present paper, with 5 to 10 cc. samples, and (b) by a macromethod in which a 100 cc. sample

TABLE IV  
*Volatile Acid and Alcohol Produced by Pathogenic Bacteria in Carbohydrate-Rich Media*

The results in Columns 3, 4, 6, and 7 are expressed as mm per liter; in Column 8 as cc. of N acid per liter.

Bacteria	Alcohol			Volatile acids			
	Procedure of present paper, oxidation to acid		Alcohol by Friedmann-Klaas method	Procedure of present paper		Macromethod, 100 cc. sample	
	K	Alcohol		K	Volatile acid (acetic)	Formic acid	Other volatile acid (acetic)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Pneumococcus, Type II..	86.2	12.2		85.6	12.7	25.2	11.7
“ “ III..	87.6	4.6		85.9	4.5	10.3	5.1
<i>Vibrio cholerae</i> .....	86.5	16.3	16.8	87.4	13.2	29.9	12.3
<i>Bacillus dysenteriae</i> , Flexner.....	86.6	13.9	13.8	88.3	14.3	27.2	14.1
<i>Bacillus typhosus</i> .....	85.7	17.9		87.6	17.2	36.6	17.3
“ <i>typhi murium</i> .....	86.5	20.2	20.4			17.4	15.6
“ <i>enteritidis</i> .....	86.8	16.7	17.0	88.6	12.8	11.2	13.5
“ <i>suipestifer</i> .....	87.0	14.3	14.8	87.6	14.0	13.3	13.5
Friedländer's bacillus.....	86.0	18.9		87.9	16.2	9.8	16.4
<i>Bacillus coli</i> .....	85.9	20.6		87.6	18.7	8.9	18.7
✓ “ <i>paratyphosus</i> B... ..	87.1	21.3	21.9	87.1	17.3	3.8	16.9
“ <i>acidi lactici</i> .....	86.0	23.6		87.1	16.6	0.6	17.1
<i>Staphylococcus aureus</i> .....	87.1	7.4		91?	0.5		
“ “.....	86.1	4.2			-0.3		
<i>Streptococcus haemolyticus</i> ..	84.5	0.3		84.9	0.7		
“ “.....	87.8	0.4		85.5	1.2		

was distilled. In the latter procedure a larger apparatus was used, 1000 cc. of distillate were collected, and the volatile acid was titrated with 0.1 N NaOH. The distribution constant (Column 5) in every instance agreed with the constant for acetic acid. Thus, of the volatile acids not removed by acid HgO, these

organisms produced only acetic acid. Most of the cultures also contained formic acid. This was determined in the distillate from the 100 cc. sample by the method of Franzen and Greve and Fincke (11). The precipitated  $\text{HgCl}$  was titrated iodometrically by the method of Utkin-Ljubowzoff (12). The results are very small and of doubtful value in the streptococcus and staphylococcus cultures; they are therefore not shown. However, in the case of the colon-typhoid organisms and the pneumococcus, which yielded larger quantities of volatile acids, the results by the procedure here described (Column 6) agree fairly well with those obtained by the macromethod (Column 8). The agreement of the results by the procedures here described with those obtained by other methods and the consistency of the results as a whole demonstrate the applicability of these procedures to complex culture media.

#### SUMMARY

Procedures are described for the identification and quantitative determination of minute quantities of volatile alcohols and acids. For a brief summary of the essential steps see the introductory portion.

The determination of alcohol in 5 cc. samples of blood yields results which agree closely with results obtained on 1 cc. samples by the method of Friedemann and Klaas. With 5 cc. samples, the minimum concentration of alcohol at which the method is still reliable is about 50 mg. per cent.

Determinations of alcohol and volatile acids in culture media were made to demonstrate the reliability of the methods. Cultures of the pneumococcus, *Vibrio cholerae*, nine organisms of the colon-typhoid group, *Staphylococcus aureus*, and *Streptococcus hemolyticus* were analyzed. The only alcohol produced by their metabolism in carbohydrate-rich media is ethyl alcohol; and the only volatile acid (not removed by  $\text{HgO}$ ) is acetic acid.

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# STUDIES IN MINERAL METABOLISM WITH THE AID OF ARTIFICIAL RADIOACTIVE ISOTOPES\*

## I. ABSORPTION, DISTRIBUTION, AND EXCRETION OF PHOSPHORUS

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Prior to 1935, studies on the absorption of phosphorus were carried out either by the use of intestinal fistulas, or by the addition of an inactive unabsorbed marker to the food, such as iron oxide in the method of Bergeim (1). Recently the excretion of calcium and phosphorus into the large intestine of the rabbit has been studied by Cowell (2) by means of the differential analysis of fecal pellets and changes in the concentrations of these elements from cecum to anus. With the discovery of induced radioactivity (3), and the preparation of the radioactive phosphorus isotope of atomic weight 32 (4), it has become possible to investigate the rates of absorption, distribution, and excretion of phosphorus, and the percentages involved. One can now distinguish the administered phosphorus from that present in the animal under investigation (5, 6).

Hevesy, in 1923, demonstrated the use of radioactive isotopes as indicators in biological investigations (7), and Chiewitz and Hevesy were the first to use the  $P^{32}$  isotope in studies of phosphorus metabolism (6, 8). Since then, with the invention and development of the "cyclotron" by Lawrence and collaborators (9, 10), and the resultant availability of "radiophosphorus," work has been published on certain phases of phosphorus storage and elimination (6, 8, 11-13), on the rate of phospholipid synthesis (14-17), and upon the locale of this synthesis (17).

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In the majority of these studies measurements were not made until 1 or more days had elapsed following the phosphate administration, at which time the phosphorus administered had reached a relatively steady state with respect to that formerly present in the animal. In only one case (13) had the rates of absorption and excretion, which occur mainly in the first 24 hours, been investigated. Accordingly, most of the present work has been confined to the changes occurring in the first 48 hours following the administration, orally or by intraperitoneal injection, of a single dose of neutral sodium phosphate, particular attention being paid to the rate and amount of excretion and the initial movements and storage of the phosphorus.

### *Methods*

The animals used were rats weighing from 220 to 300 gm. and falling into two age groups of 70 to 100 and 100 to 130 days, respectively, at the time of use. Each animal was fasted 12 hours before being given the dose of phosphate. 1 or 2 hours after administration of the phosphate the rat was given access to food.

The radiophosphorus was prepared from ordinary red phosphorus by bombardment with deuterons in the cyclotron of the Radiation Laboratory of the University of California. After the bombardment the sample was digested with dilute HCl to remove traces of aluminum, filtered, and then oxidized to  $\text{H}_3\text{PO}_4$  with 8 N  $\text{HNO}_3$ . This was evaporated on a steam bath and the excess  $\text{HNO}_3$  decomposed with concentrated HCl. The solution was again evaporated, diluted, and neutralized with NaOH (heavy metals, if present, were precipitated with  $\text{H}_2\text{S}$ ) to a pH of about 8; *i.e.*, acid to phenolphthalein and alkaline to litmus. The resultant phosphate solution was made up to such a concentration that 1 ml. contained around 1000 to 2000 times the minimum quantity of  $\text{P}^{32}$  which could be detected on the electroscope. This volume constituted a single dose. The amount of phosphate given per dose was therefore dependent upon the relative radioactivity of the sample used and the sensitivity of the measuring device. In these experiments the dose varied from 4.5 to 13 mg. of phosphorus.

After administration of the phosphate, each animal was placed in a separate cage over a urine-feces separator (18). At the end

of the desired period, measured from the time of administration, the animal was anesthetized with chloroform and bled by heart puncture, following which the tissues to be examined were dissected out. These tissues and the urine, feces, and blood samples were then ashed with  $\text{Mg}(\text{NO}_3)_2$  at  $500^\circ$  (19). The ash was dissolved in  $\text{HCl}$  or  $\text{HNO}_3$ , and the phosphate in an aliquot containing about 5 mg. of P was precipitated, first as ammonium phosphomolybdate, then as magnesium ammonium phosphate (19). The  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  was filtered through a Whatman No. 50 filter paper, dried at  $120\text{--}140^\circ$ , and transferred quantitatively to a  $3 \times 4$  cm. copper tray. The filter paper was ashed and added to this and the whole was then spread uniformly over the surface of the tray. This precaution, and the use of 5 mg. aliquots of phosphorus in each case, kept the error due to self-absorption of  $\beta$ -rays at a low, and nearly constant level, for the thickness of the measured powder was only a fraction of a mm. Radioactivity was measured by means of a Lauritsen electroscope in which an aluminum foil window had been inserted. Aliquots of the solution of phosphate administered, equal in phosphorus content to the unknown samples, were analyzed in the same manner for  $\text{P}^{32}$ , and served as the standard of comparison for the unknown samples.

The radioactivity was measured in arbitrary units and, after correction for decay, is expressed in terms of the per cent of the radioactivity of the administered phosphate. Total phosphorus, when it was measured, was determined on the ash by the colorimetric method of Fiske and Subbarow (20), as modified by King (21).

The experiments carried out here were divided into four series. Series I consisted of sixteen rats of the older group which were given the phosphate by stomach tube. Series II consisted of eight rats of the same age group which received the phosphate by intraperitoneal injection. In Series III four rats of the younger group were given the phosphate by stomach tube. Series IV was a study of excretion in both age groups, the phosphate being administered by stomach tube, by intraperitoneal injection, or mixed with food. Urine and feces were collected at short intervals and analyzed separately for about 5 days thereafter.

Rats in Series I received 6 to 13 mg. of P, the majority (ten out of sixteen) receiving 12 mg. of P. The animals in Series II and

III received 4.5 mg. of P (a stronger sample of  $P^{32}$  was used here), while those in the experiments on excretion in Series IV received from 4.5 to 28 mg. of P.

### Results

*Absorption*—In Fig. 1 is plotted the percentage of unabsorbed phosphorus found in the gastrointestinal tracts of the rats of Series I and III. In Series I the rats were given access to food

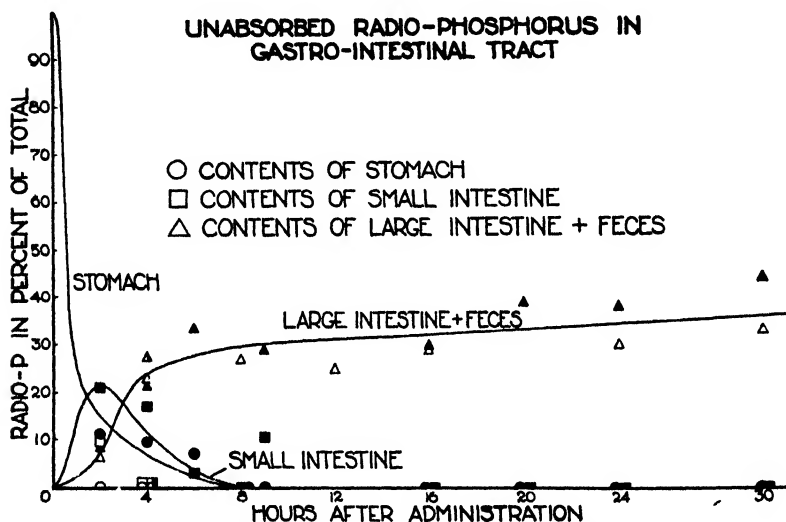


FIG. 1. Residual radiophosphorus in the gastrointestinal tract after oral administration. The solid symbols represent those animals given access to food 1 hour after administration of the phosphate dose; the open symbols those for which 2 hours elapsed.

1 hour after administration of the phosphate, which may or may not be the reason for the delayed absorption as compared to the rats of Series III where 2 hours elapsed.

The unabsorbed phosphorus was calculated by subtracting the amounts found in each section of the gut of animals which had been injected intraperitoneally with the same amount of phosphate and killed following the same time interval. In other words, the values in Series II (varying from 0.5 to 2 per cent) were subtracted from the corresponding values in Series I and

III in order to correct the latter for radiophosphorus secreted or excreted into the intestines from previously absorbed material.

The major portion of the absorption occurs within the first 2 hours and the stomach is usually empty of radiophosphorus within 4 hours. In no case was any unabsorbed radiophosphorus found in the stomach at or after 8 hours. The small intestine naturally is cleared more slowly of phosphorus, but in only one case (at 9 hours) was any unabsorbed radiophosphorus found in the small intestine after 8 hours.

Although not yet appearing in the feces, radiophosphorus is found in the large intestine and cecum within 2 hours after administration. These figures also represent only unabsorbed material, having been corrected for the amount excreted from the blood into the large intestine as estimated from Series II. Within 4 hours after ingestion, the major portion of the administered radiophosphorus not retained by the tissues has found its way into the urine and contents of the large intestine.

*Distribution*—The major portion of the absorbed phosphorus is taken up by the muscle and bone, as might be expected from their relative mass and high phosphorus content, respectively. The amounts involved could not be estimated very accurately owing to the errors involved in sampling and measuring a small aliquot. Muscle, on the basis of the analysis of one gastrocnemius in each animal, and assuming a muscle content of 43 per cent of the body weight (21), took up about 15 per cent in 4 hours. Subsequently the amount diminished slowly to about 10 to 12 per cent in 150 hours. From femur analyses, assuming this to represent the 6.1 per cent of bone in the rat (21), bone attains a value of 20 to 25 per cent in 2 to 4 hours, dropping to around 15 per cent after 150 hours. After 4 to 8 hours the carcass, by which is meant the residue after removal of the viscera and their contents but including both muscle and bone, contained a maximum of about 60 to 65 per cent of the absorbed phosphorus, following which there is a drop to 50 or 55 per cent in 48 hours.

Among the viscera, liver takes up the largest fraction of the absorbed phosphorus, followed by the stomach and small intestine (considered together). The accumulation and withdrawal from these organs are shown in Fig. 2 in terms of per cent of phosphorus injected intraperitoneally (curve *IP*) and per cent of phos-



phorus given by stomach tube (curve *ST*). When the latter values are corrected for the amounts not absorbed from the intestinal tract, they approach the values found in the injection series. Recent work has shown that the small intestine accounts for most of the total.

Over half of the phosphorus accumulating in the liver in the first 6 hours is in the form of newly synthesized phospholipid, as shown by Perlman, Ruben, and Chaikoff (17). Only a very

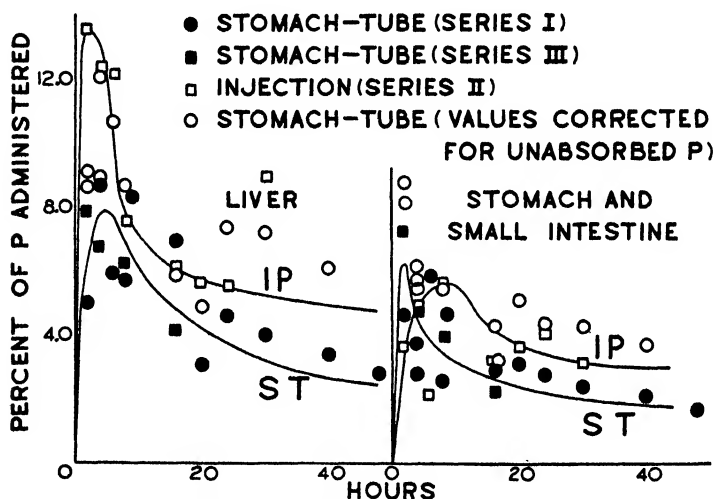


FIG. 2. Retention of radiophosphorus in liver and in stomach and small intestine when given orally (*ST*) and when injected intraperitoneally (*IP*). The open circles represent the points of Series I and III in terms of per cent of absorbed radiophosphorus.

small portion of this can be due to the blood in the liver, as the blood at no time contains more than 4 per cent of the total radiophosphorus in its entire volume.

Stomach and small intestine show differently shaped curves for the two types of administration. The sharp rise and abrupt fall obtained when the phosphate is given by stomach tube may be explained by the superimposition of absorption upon accumulation. This may also explain the very high values found during the period of 2 to 4 hours when the figures are expressed in terms of the amount of phosphorus absorbed as compared to the data

from the injection method. In these tissues, as in liver, a large part of the radiophosphorus found is in the form of phospholipid (17).

The per cent of absorbed radiophosphorus found in the blood varied inconsistently from 2.6 to 4.0 per cent after 2 hours to around 1 per cent after 48 hours had elapsed.

In Fig. 3 are plotted the radiophosphorus contents of kidneys, lungs, heart, and brain. Owing to the small amounts no high

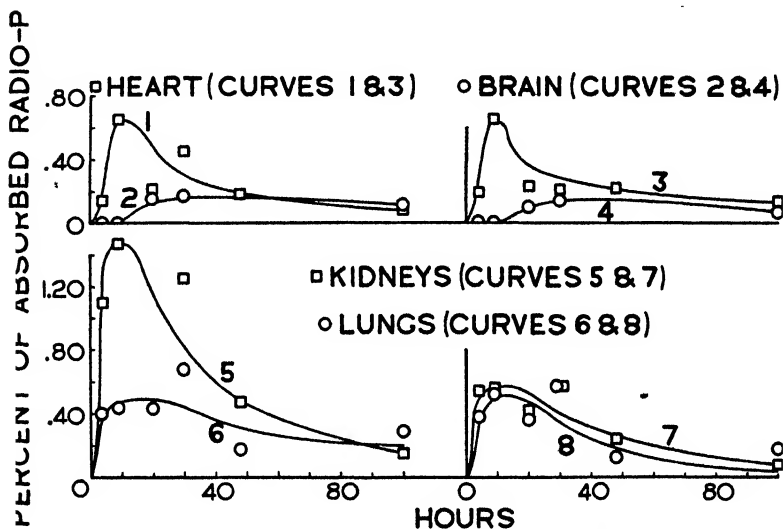


FIG. 3. Retention of radiophosphorus by kidneys, lungs, heart, and brain in per cent of absorbed radiophosphorus. Curves 1, 2, 5, 6, radiophosphorus in whole organ; Curves 3, 4, 7, 8, radiophosphorus per gm. of fresh tissue.

degree of accuracy is claimed for these measurements; they are included to show the general trends and approximate affinities of these organs for absorbed phosphate. Of interest are the retentions of phosphorus by kidney and brain. The former shows a rapid rise and a fairly rapid fall in radiophosphorus content, but the fall is not so rapid as is that of the urinary excretion. Brain shows a lag in accumulation and a very slow turnover of phosphate, a fact noted by Artom *et al.* (14).

More important than the phosphorus retention of the whole

of a particular tissue or organ is the "specific affinity," *i.e.*, the retention (in per cent of absorbed radiophosphorus) per gm. of the fresh tissue. Only when the foregoing figures have been reduced to this basis, can the retentions of the different tissues be rightly compared. These are given in Figs. 3 and 4.

As noted in the cases of kidneys, lungs, heart, and brain, the figures given for bone, muscle, and carcass are significant only as an indication of the order of magnitude of the phosphorus retention of these tissues.

Skin, including hair and adherent subcutaneous tissue, showed a slow rise from a content of 0.05 to 0.09 per cent of absorbed radiophosphorus per gm. in 40 hours.

Bone shows the largest specific affinity of any tissue studied, although, when reduced to unit phosphorus content, it becomes the lowest. Liver, stomach and small intestine, heart, kidneys, lungs, carcass, blood, muscle, and brain show decreasing specific affinities in this order. The typical sharp rise and gradual fall in radiophosphorus content are observed in all cases with the exception of brain.

*Excretion*—In Fig. 5 are plotted the radiophosphorus contents of the urine and feces for the rats in Series I to IV in per cent of the total administered. Curves 1, 2, and 3 represent the per cent excreted in the urine under the various conditions up to the times noted. Curves 4 to 8 show the corresponding fecal excretions.

Curve 1 is drawn to cover two series of points, representing 4.5 and 9 mg. of P, respectively, injected intraperitoneally. The 9 mg. points are the averages of two animals in Series IV. The 4.5 mg. points are the averages of one animal in Series IV and the eight individual rats in Series II.

Curve 2 represents the urinary excretion, in per cent of total radiophosphorus administered, for the same amounts given by stomach tube. The 4.5 to 6 mg. of P points represent the averages of experiments on one rat of Series IV and four rats of Series I (6 mg. of P) run continuously in the same manner as those in Series IV. The 9 mg. points are the averages found for two rats in Series IV. One curve fits both series of points and this curve, if corrected for the amount unabsorbed (average = 30

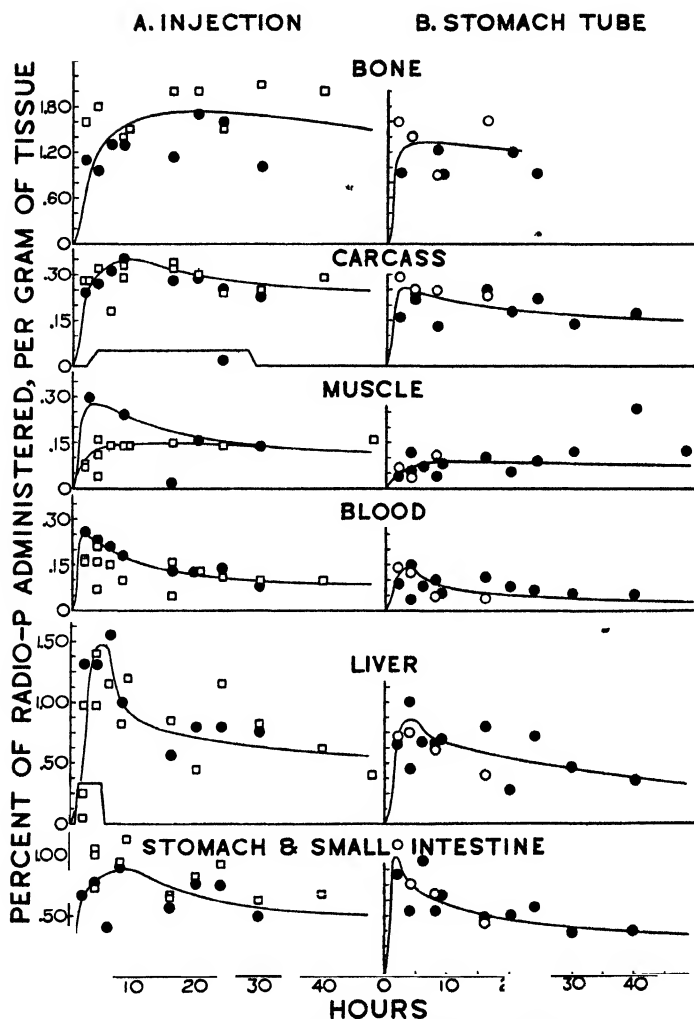


FIG. 4. Retention of radiophosphorus in various tissues and in blood per gm. of fresh weight. *A*, administered by intraperitoneal injection; ● Series II, □ Series I and III (stomach tube) in terms of per cent of absorbed radiophosphorus. *B*, administered by stomach tube; ● Series I (6 to 13 mg. of P), ○ Series III (4.5 mg. of P).

per cent; factor = 1.4), will approach the injection curve quite closely.

Curve 3 represents urinary excretion in two rats of Series IV following the administration of 28 mg. of P in 3 gm. of normal rat food. It rises a bit more slowly owing to slower absorption, but approaches Curve 2 as time increases.

Curve 4 represents the per cent of administered phosphate found in the large intestine, cecum, and feces together. Each point is the average for two rats, one of Series I and one of Series

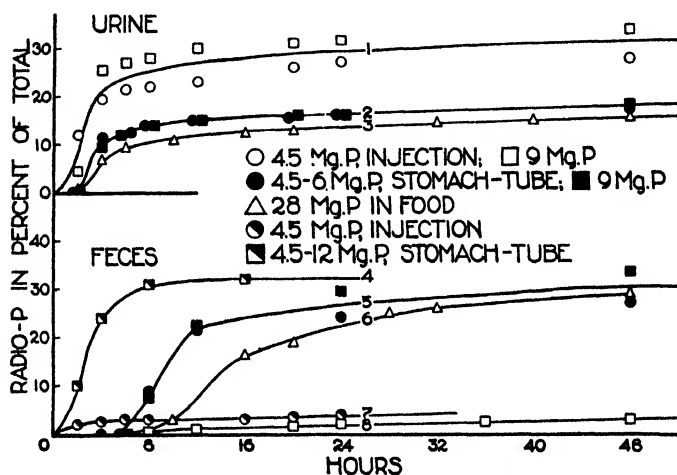


FIG. 5. Excretion of radiophosphorus following administration of phosphate orally or by intraperitoneal injection. Curves 1, 2, and 3, urinary excretion; Curves 5, 6, and 8, fecal excretion; Curves 4 and 7, total amounts in large intestine, cecum, and feces.

III. Each individual value is within one unit of the average value given.

Curve 5 is drawn from the average of the values obtained from two rats in Series IV, given 4.5 and 9 mg. of P, respectively, and the averaged values of four rats in Series I (6 mg. dose) measured continuously. This curve approaches the plateau of Curve 4 more slowly, as it represents feces only.

Curve 6 is the fecal excretion of the two rats given the radiophosphorus in food and, as expected, shows a still slower rise to the maximum.

Curve 7 represents the sum of the amounts found in large intestine, cecum, and feces in the rats of Series II, injected intraperitoneally with 4.5 mg. of P. Each point represents a separate rat.

Curve 8 represents the averaged values of three rats of Series IV, two of which were given 9 mg. of P intraperitoneally, the third receiving 4.5 mg. of P in the same manner.

The large fecal excretion of ingested phosphorus, shown in Curves 4, 5, and 6, represents mainly unabsorbed phosphorus, for the 70 per cent which was absorbed would follow the same course as the 100 per cent injected and would give the same small amount of radiophosphorus in the feces shown in Curves 7 and 8. However, it cannot be said that excretion or secretion of absorbed or injected phosphate into the intestine and its subsequent excretion via the feces is negligible, a hypothesis that may be suggested by the latter two curves. Once absorbed into the blood, the radiophosphorus administered marks not only the 4.5 or 9 or 12 mg. of phosphorus associated with it at the start, but a larger amount limited only by the total phosphorus in the body. In other words, the active phosphorus becomes "diluted" with inactive phosphorus in the blood and tissues, and we can then no longer say that 2 per cent of the administered active phosphorus represents 2 per cent of 4.5 or 9 or 12 mg. of phosphorus. Until both this dilution and that of unabsorbed  $P^{32}$  due to digestive juices are measured, no estimate of the relative amount of phosphorus in the feces due to lack of absorption and that due to excretion from the organism may be made.

Although we cannot yet place an absolute value on the fecal excretion of absorbed phosphorus, we can determine the ratio of urinary to fecal excretion of this material directly from the curves. Assuming that both urine and intestinal secretions are removed from the blood at about the same rate and time, the ratio of  $P^{32}/P^{31}$  will be the same in each. Therefore the ratio  $P_f^{31}/P_u^{31}$  for absorbed phosphorus (where  $P_f^{31}$  = total P added to the contents of the large intestine during the interval measured, and  $P_u^{31}$  that added to the urine) will equal the ratio  $P_f^{32}/P_u^{32}$ , which is shown by the values of Curves 8 or 7 over those of Curve 1 (Fig. 5). At 48 hours this ratio is about 1:10. That is to say, only about 1/11 of the absorbed phosphorus excreted by the body

in this time is eliminated via the gut and feces, while 10/11 is excreted via the urine. This is in fair agreement with the ratio found by Chiewitz and Hevesy (8) in humans. Furthermore, if the ratio  $P_f^{31}/P_u^{31}$  is known, the determination of  $P_u^{31}$  allows  $P_f^{31}$  to be calculated and a definite value thus to be placed on the truly excreted fecal phosphorus. In like manner the absolute phosphorus uptake or turnover of an organ or tissue in any given length of time may be calculated.

Worthy of note are the rapid rises in Curves 1, 2, 3, 4, and 7, showing that the major portion of the administered phosphorus not retained by the tissues finds its way into the large intestine or urine within the first 4 to 8 hours, subsequent elimination being very slow. These facts, coupled with those shown by the retention curves, support our starting hypothesis that the first 24 hours following administration are the most important ones for studying the movements of a single dose of phosphorus.

#### SUMMARY

1. Study of phosphorus metabolism in rats with the aid of radiophosphorus has shown that the major disposition of injected or ingested phosphate occurs within the first 8 hours after administration.

2. Absorption of phosphorus, administered as dissolved  $\text{Na}_2\text{-HPO}_4$ , is most rapid in the first 2 hours and is usually at an end 8 hours after ingestion. The absorption is never complete, about 30 to 40 per cent of the ingested phosphorus remaining unabsorbed.

3. Of the injected or absorbed phosphorus about 20 to 30 per cent is excreted in the urine within 8 hours, while about 3 per cent is excreted via the large intestines. The subsequent elimination of this phosphorus over a 5 day period in both urine and feces goes on at a steadily diminishing rate that amounts to about 2 to 1 per cent per day.

4. The tissues which were examined retain the absorbed or injected phosphorus in the following decreasing order: bone, muscle, liver, stomach plus small intestine, blood, kidneys, heart, lungs, and brain. All except brain show a rapid uptake in the first 10 hours, followed by a prolonged, steadily diminishing loss. Brain apparently has a very slow turnover of phosphorus.

5. Per unit of fresh weight of tissue the retentions—called the “specific affinities”—assume the following order: bone, liver, stomach plus small intestine, heart, kidneys, lungs, blood, muscle, skin, and brain.

Our thanks are due to several individual members of the staff of the Radiation Laboratory, to Mr. S. Ruben, and to Mr. M. Joseph for their advice and assistance in the course of these experiments. The work was also aided by the Works Progress Administration.

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# THE ANALYSIS OF CALCIUM IN BLOOD AND OTHER BIOLOGICAL MATERIAL BY TITRATION WITH CERIC SULFATE

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The advantages of ceric sulfate over permanganate in oxidimetric titrations are now sufficiently well known so that they need not be dwelt upon here.

A method for the estimation of calcium in which the calcium oxalate is oxidized with a measured quantity of ceric sulfate and the excess titrated with ferrous ammonium sulfate (Mohr's salt) in the presence of *o*-phenanthroline as indicator has been used in this laboratory for the past 2 years with advantage over the methods previously employed. The titration is carried out at room temperature, and there is no blank if the *o*-phenanthroline, which is in the reduced form, is oxidized before being used.

A procedure for the estimation of calcium in blood serum by a combined ceric sulfate and iodine titration has been published by Rappaport and Rappaport (1). Katzman and Jacobi (2) have developed a direct ceric sulfate titration by employing iodine monochloride as a catalyst. We believe the method given here is superior to the above in certain respects.

## *Reagents—*

Saturated ammonium oxalate (about 4 gm. per 100 ml. of  $H_2O$ ).

2 per cent ammonium hydroxide solution (2 ml. of concentrated  $NH_4OH$ , 98 ml. of  $H_2O$ ). This is best saturated with calcium oxalate by agitating with a small portion of the solid calcium oxalate and filtering off the undissolved material.

0.01 M ceric sulfate. Dissolve 13.2 to 13.4 gm. of anhydrous  $Ce(SO_4)_2$  by heating with 20 ml. of concentrated  $H_2SO_4$  and adding water until dissolved. Stir during this process. Transfer

the contents to a 1 liter volumetric flask and dilute to the mark. Standardize the solution with sodium oxalate or ferrous ammonium sulfate. The ceric sulfate solution should be kept from contact with rubber stoppers or other carbonaceous material.

0.005 M ferrous ammonium sulfate (Mohr's salt). Dissolve 1.96 gm. of the above with 9 ml. of concentrated HCl and make up to 1 liter.

Phenanthroline indicator. Dissolve 0.695 gm. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  in water in a 100 ml. volumetric flask. Add 1.485 gm. of *o*-phenanthroline monohydrate,<sup>1</sup> stir until dissolved, and make up to volume. Before this indicator is used, titrate 1 ml. of it to the neutral point (purple) with the ceric sulfate solution. Use 2 drops of the neutralized indicator for a titration.

#### *Procedure*

To 2 ml. of serum or an aliquot of liquid containing between 0.1 and 0.4 mg. of calcium, add 2 ml. of  $\text{H}_2\text{O}$  and 1 ml. of the saturated ammonium oxalate. Let stand for 2 or more hours. If a trichloroacetic acid filtrate of blood serum or the acid extract of ashed tissue is used, first neutralize the free acid of the filtrate to the pH of about 5.0 by adding KOH or  $\text{NH}_4\text{OH}$  solution until a blue-green color is obtained with the indicator brom-cresol green.

After the precipitate has stood sufficiently long, filter through a Kirk-Schmidt (3) microfilter which has a stem 2 inches long. Wash twice with 3 ml. portions of 2 per cent  $\text{NH}_4\text{OH}$  solution, pouring the washings from the precipitation tube onto the filter.

Now place the precipitation tube within the suction flask and insert the tip of the filter within it. Dissolve the calcium oxalate precipitate from the filter pad with three 1 ml. portions of hot 2 N  $\text{H}_2\text{SO}_4$ . Then wash out the residue with two portions of 2 to 3 ml. each of water.

To titrate add 2 ml. of 0.01 M ceric sulfate to the test-tube containing the dissolved calcium oxalate and let stand for 30 minutes. Now add 2 drops of neutralized phenanthroline and titrate back with ferrous ammonium sulfate to the end-point. The color changes in the titration are purple→blue-green→blue→salmon.

<sup>1</sup> Purchased from the G. Frederick Smith Chemical Company, Columbus, Ohio.

At the end-point the change is from blue to salmon upon the addition of 0.001 ml. of 0.01 N ferrous ammonium sulfate.

The titrations can be carried out rapidly, since in the region from purple to blue no particular care need be used. When the blue stage is reached, the remainder of the titration can be accurately watched to attain the exact end-point.

The calcium value is simply calculated, since each ml. of 0.01 M ceric sulfate used up in the titration is equivalent to 0.4 mg. of calcium.

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## THE METABOLISM OF SULFUR

### XXV. DIETARY METHIONINE AS A FACTOR RELATED TO THE GROWTH AND COMPOSITION OF THE HAIR OF THE YOUNG WHITE RAT.

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(Received for publication, November 29, 1937)

The relation of the cystine of the diet to the growth and composition of the hair of young white rats has been established (1-3). In the absence of adequate amounts of dietary cystine, optimal growth of the hair was not observed and the cystine and sulfur contents of the hair were lower than those of rats maintained on diets supplying ample amounts of cystine. The ingestion of cystine in quantity in excess of the normal physiological requirements, however, failed to result in the production of a "superkeratinized" hair (3).

Methionine has been shown to function similarly to cystine, both for purposes of growth in rats fed a diet inadequate in its content of cystine (4, 5) and for the detoxication of monobromobenzene (6-8). The administration of either cystine or methionine to bile fistula dogs was followed by an augmented excretion of taurocholic acid in the bile (9). When methionine was fed to cystinuric individuals, an increased excretion of cystine by the kidneys was observed (10, 11). The evidence available thus indicates a close relationship in metabolism between the two chief sulfur-containing amino acids of the protein molecule. Rose (12) has suggested that methionine may replace cystine of the diet entirely for purposes of growth of the young white rat, but that there is a minimal dietary requirement for methionine and that cystine cannot be substituted effectively for all of the dietary methionine.

In view of these facts, it has seemed important to determine

whether methionine, as a supplement added to a cystine-deficient diet, is able to function similarly to cystine and to lead to the production of hair of normal cystine and sulfur content in young white rats.

#### EXPERIMENTAL

Seven litters of young white rats of approximately 60 gm. of weight were placed in individual cages and maintained on a basal diet which had been shown to be deficient in its content of cystine and to be adequate for good growth, when supplemented by cystine or methionine (4). The composition of this diet was as follows: corn-starch, 54.7 per cent; lard, 25.0 per cent; whole milk powder, 15.0 per cent; gelatin, 2.0 per cent; sodium chloride, 1.7 per cent; Osborne and Mendel salt mixture (13), 1.0 per cent; vitamin B adsorbate,<sup>1</sup> 0.6 per cent. In addition, 100 mg. of cod liver oil and 125 mg. of dried yeast were fed separately to each animal daily. After maintenance for a week (in the case of Litters B and C, 5 weeks) on the basal diet, the animals were divided into two groups. For the control group, which was to be continued on the basal diet, the animals selected were those which were heavier, which had consumed the basal diet most readily, and which had made the best gains in weight during the preliminary experimental period. The other group received the basal diet, supplemented by the addition of 4.5 gm. of *dl*-methionine per 1000 gm. of food.

Litters A to C were permitted unrestricted consumption of food for 31 to 42 days, the animals being weighed at regular intervals and a careful record of food consumption being made. With the other four litters, the experimental method of restricted diet or paired feeding was employed, the amount of food received daily by the animal on the basal diet supplemented by methionine being determined by the previous day's food consumption of a paired litter mate of the same sex, which was fed the basal diet. The durations of the experimental periods for Litters D to G were 56, 40, 39, and 42 days respectively.

At the conclusion of the experimental periods, the rats were

<sup>1</sup> We wish to express our indebtedness to Eli Lilly and Company of Indianapolis for the generous gift of the vitamin B adsorbate used in these experiments.

chloroformed and as much as possible of the hair was removed by shaving without the use of soap or water. The hair was then extracted with absolute alcohol and chloroform for 48 and 18 hours respectively (14), air-dried for 24 hours, and placed in a desiccator over calcium chloride until the analyses were completed. The weight of the hair recorded in Tables I and III is the weight after extraction and drying. \*

All determinations of total sulfur were made according to the Benedict-Denis procedure, as modified for use in this laboratory (15), by a preliminary digestion of the hair for 6 hours with concentrated nitric acid. Attempts to determine the sulfur content of the methionine by the unmodified Benedict-Denis method proved unsatisfactory. Ten series of determinations, all in triplicate, gave results which ranged from 7.97 to 16.98 per cent, with an average value of 13.34 per cent of sulfur as compared with the theoretical value of 21.49 per cent. The recovery of sulfur was only slightly improved when the preliminary digestion with nitric acid was included. The results of the Parr bomb method were used as a further check in the determination of the purity of the methionine used in the feeding experiments. Determinations by the Parr bomb method gave essentially theoretical values for the sulfur of the methionine.

Shortly after these results were obtained, similar difficulties in the determination of the sulfur of methionine were reported by Painter and Franke (16) and later by Rutenber and Andrews (17). They concluded that the Benedict-Denis method is not reliable for the determination of total sulfur in proteins and that the error is chiefly due to the abnormal behavior of methionine. Since the methionine sulfur of hair is low in comparison with the cystine sulfur, it was felt that the use of the *modified* Benedict-Denis method should not seriously affect the values obtained for the total sulfur of hair and the use of this more convenient method was continued. It is possible that the sulfur of methionine, when this amino acid is in combination in the protein molecule, may be more accurately determined by this procedure than the sulfur of the free amino acid. In order to satisfy ourselves that the use of the modified Benedict-Denis procedure did not introduce any serious error into our analyses of hair, we have analyzed a number of our samples of rat hair by the Parr bomb method as well as



by the modified Benedict-Denis procedure. The values were essentially the same by both methods. Moreover, in a considerable number of unpublished parallel analyses of cow hair in this laboratory, no evidence was obtained that the Parr bomb method gave higher values than the modified Benedict-Denis method.

Cystine was determined by the Sullivan-Lugg procedure (18) with the modification of the time factor suggested by Roussouw and Wilken-Jorden (19).

#### DISCUSSION

Detailed data from a typical litter unit of each of the types of feeding experiments are presented in Tables I and II. In confirmation of the work of previous investigators (4, 5), who employed diets similar to our own, addition of methionine to a diet known to be low in its cystine content resulted in increased growth, which was evident when calculated either as the daily gain or as the gain per 100 gm. of food eaten. This result has been obtained without exception with four litters (thirty-five rats) in paired feeding experiments and with three litters (twenty-six rats) whose diet was unrestricted in amount. The values for the complete series of animals are summarized in Table III.

The amount of hair per 100 gm. of rat was invariably somewhat higher in those animals receiving supplementary methionine, although in a few instances, particularly in the paired feeding experiments, the differences were scarcely beyond experimental error (*cf.* Pair XV, Table I). The total sulfur and cystine contents of the hair produced when methionine supplemented the diet were greater than those of the hair of control animals on the basal diet alone. These results are quite similar to those of earlier experiments (1-3), in which cystine supplemented a basal diet low in cystine.<sup>2</sup> The composition of the hair is comparable to that of young white rats which received standard mixed diets or diets of high protein content, as previously determined in this laboratory (1).

The tendency toward an increased production of hair after

<sup>2</sup> It should be noted, however, that the basal diet, the treatment of the hair prior to analysis, and the method used for the determination of cystine in the present series differed from those of the earlier investigations. For this reason, detailed comparison with our earlier studies is not justified.

TABLE I

*Growth of Young White Rats and Composition of Hair As Influenced by Addition of Supplementary Methionine to Cystine-Deficient Diet in Paired Feeding Experiments (Litter G)*

The animals were paired as to sex and food intake. Except for Pair XIII, the animals were males. The duration of the experimental period was 42 days. Diet B signifies the basal diet; B + M, the basal diet supplemented with 0.45 per cent of methionine.

Pair No.	Rat No.	Diet	Supplementary methionine for period	Gain		Hair		
				Average daily	Per 100 gm. food	Per 100 gm. rat	Total S	Cystine
			gm.	gm.	gm.	gm.	per cent	per cent
XIII	52	B		0.6	8.6	0.75	3.73	11.99
	53	" + M	1.31	1.2	17.7	1.04	4.21	13.50
XIV	54	"		0.5	6.8	0.60	3.60	11.29
	55	" + M	1.32	1.3	18.7	0.87	4.02	12.68
XV	56	"		0.4	8.3	0.87	3.57	11.31
	57	" + M	0.99	0.9	16.5	0.90	3.98	12.68
XVI	58	"		0.8	10.3	0.71	3.79	12.44
	59	" + M	1.37	1.4	19.7	0.86	4.21	13.58

TABLE II

*Growth of Young White Rats and Composition of Hair As Influenced by Addition of Supplementary Methionine to Cystine-Deficient Diet in Unrestricted Feeding Experiments (Litter B)*

The duration of the experimental period was 39 days. Diet B signifies the basal diet; B + M, the basal diet supplemented by methionine.

Rat No.	Sex	Diet	Supplementary methionine for period	Food average daily	Gain		Hair	
					Total	Per 100 gm. food	Total S	Cystine
			gm.	gm.	gm.	gm.	per cent	per cent
10	F.	B		9.5	33.1	8.9	3.66	12.65
11	M.	"		7.9	22.2	7.1	3.96	12.54
12	F.	"		7.4	29.3	10.1	3.63	12.47
13	"	"		7.3	23.5	8.2	3.87	12.70
15	"	"		9.7	36.3	9.6	3.72	12.97
14	M.	" + M	2.04	12.2	104.9	22.0	4.24	14.17
16	"	" + "	2.13	12.1	83.7	17.7	4.08	13.64
17	F.	" + "	1.84	10.2	67.9	16.6	4.29	14.71

supplementary dietary methionine is not in accord with the single observation on a sheep reported by Marston (20). After a daily injection of 0.65 gm. of methionine into a Merino ewe over a period of 10 days, he observed a slight increase (5 per cent) in wool production, an increase which he considered insignificant,

TABLE III

*Growth, Production of Hair, and Composition of Hair of Young White Rats Fed a Basal Cystine-Deficient Diet and Same Diet Supplemented with Methionine*

Two groups are presented, Litters A to C, which were permitted unrestricted food consumption, and Litters D to G, in which the paired feeding method was used. The values represent the averages for all animals. Diet B signifies the basal diet; B + M, the basal diet supplemented with 0.45 per cent of methionine.

	Diet	Unrestricted feeding (Litters A, B, C)		Paired feeding (Litters D, E, F, G)	
		Male	Female	Male	Female
No. of animals	B	6	8	9	8
	" + M	7	5	11	7
Average daily gain, gm.	"	0.72	0.71	0.61	0.69
	" + M	2.15	1.62	1.24	1.32
Average daily food intake, gm.	"	6.8	8.0	6.8	7.1
	" + M	9.9	8.6	6.5	6.9
Average gain per 100 gm. food, gm.	"	11.1	9.1	10.1	9.9
	" + M	22.1	18.7	19.5	19.2
Hair per 100 gm. body weight, gm.	"	0.98	0.98	0.81	0.83
	" + M	1.30	1.24	0.94	0.91
Total S, per cent	"	3.88	3.84	3.78	3.89
	" + M	4.16	4.37	4.09	4.23
Cystine, per cent	"	12.18	12.42	11.97	12.23
	" + M	13.21	13.72	12.92	13.57
Cystine S	"	83.9	87.2	84.3	83.8
$\frac{\text{Total S}}{\text{Total S}} \times 100$	" + M	84.9	84.0	84.3	86.2

particularly since in a subsequent period of similar duration the injection of an equivalent amount of cysteine led to a 16 per cent increase. It should be noted that we were concerned with a different species, with small animals which were fed a more carefully controlled diet in which deficiency in cystine had been demonstrated, and that the amount of methionine administered

per unit of body weight must have been significantly greater than in Marston's single experiment.<sup>3</sup> In our experiments with unrestricted food consumption, the weights of hair produced when methionine was added to the diet were approximately 32 and 26 per cent greater with male and female rats respectively, and in the paired feeding series, similar increases of 16 and 10 per cent were obtained (Table III). "

The interpretation of our findings is not simple. Our basal diet contained both cystine and methionine. Methionine is an essential amino acid which cannot be replaced by cystine, as shown by Rose and his coworkers (12). However, if the minimal requirements for methionine are supplied by the diet, the rest of the needed sulfur-containing amino acids may be furnished by either cystine or methionine (12). Since our basal diet could be effectively supplemented by cystine, it would appear that this diet contained an amount of methionine adequate for minimal growth requirements, but that the diet did not furnish the requisite amount of *total sulfur-containing amino acids*. This deficiency could be remedied by either cystine (4) or methionine. Supplementary dietary methionine allowed growth of a hair of normal cystine content. Are these experiments to be interpreted as direct proof of the transformation of methionine to cystine in the living organism? Has the administration of methionine made possible the utilization of dietary cystine for the growth of hair, while methionine has taken over some other biological function of cystine? Our experiments fail to offer definite proof of either of these possibilities. Such proof could be obtained by feeding young white rats diets, whose entire amino acid content is known exactly and which contain no cystine. If, under these experimental conditions, a hair of normal cystine content is produced, the relation of methionine to keratinization (cystinization) of the hair will have been demonstrated conclusively.

#### SUMMARY

Young white rats were fed a basal diet known to be deficient in its content of the sulfur-containing amino acids (4). When methionine was added to this diet, good growth was obtained.

<sup>3</sup> The weight of the ewe is not directly stated, but it is implied that the weight was approximately 40 kilos.

The addition of supplementary methionine to the basal diet resulted in a slightly increased production of hair. This hair contained a higher percentage of total sulfur and of cystine than the hair of animals receiving the basal unsupplemented diet.

These experiments offer further evidence of the close relationship in metabolism between the two chief sulfur-containing amino acids of the protein molecule.

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## COMPARATIVE STUDIES OF THE METABOLISM OF THE AMINO ACIDS

### VII. EXPERIMENTAL ALCAPTONURIA IN THE WHITE RAT\*

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Among the unsolved problems, which are related to amino acid catabolism, is the status of homogentisic acid in the intermediary metabolism of the aromatic amino acids, tyrosine and phenylalanine. This acid has long been recognized as a constituent of the urine in the inborn error of metabolism, alcaptonuria. Dakin (1) has maintained that homogentisic acid is not a normal product of intermediary metabolism but that it originates in a series of reactions distinct from the normal and characteristic of the alcaptonuric organism. Garrod ((2) p. 73), in reviewing the evidence, has championed the theory of Neubauer (3), which postulates that homogentisic acid is formed in the catabolism of the naturally occurring aromatic amino acids and is destroyed completely by the normal organism. In alcaptonuria, the defect is believed to lie in a failure of complete oxidation of homogentisic acid, which results in the excretion of this acid by the kidneys. Dakin based his conclusions on the inability to produce alcaptonuria experimentally in the normal animal by the administration of phenylalanine or tyrosine. In the alcaptonuric patient, on the other hand, ingestion of either of the aromatic amino acids is followed promptly by an increased urinary excretion of homogentisic acid. The consistent successful demonstration of an experimental alcaptonuria produced by feeding tyrosine or

\* A preliminary report of this investigation was presented before the Thirty-first meeting of the American Society of Biological Chemists at Memphis, April, 1937 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **119**, p. lxxvi (1937)).

phenylalanine to normal animals would be important evidence in support of the theory of Neubauer (3).

Most important of the attempts to produce alcaptonuria in normal animals have been the studies of Dakin (4, 5) and of Abderhalden (6). The former injected 6 to 8 gm. of phenylalanine into cats (4) and fed similar amounts of tyrosine to cats (5). Despite the fact that the power of the organism to catabolize amino acids normally had been overtaxed, as demonstrated by the excretion of considerable amounts of the ingested amino acids by the kidneys, no homogentisic acid was detected in the urine. Abderhalden (6), after unsuccessful attempts to produce an experimental alcaptonuria in dogs and rabbits, fed the aromatic amino acids to human subjects. In one individual, in a single unconfirmed experiment, 0.5 gm. of lead homogentisate was isolated from the urine after the oral administration of 50 gm. of tyrosine. The purity of the lead salt was evidenced by its melting point and elementary analysis. Further experiments with other subjects, in one of which 150 gm. of tyrosine were fed, did not afford further evidence of experimental production of alcaptonuria.

It seemed possible that continued ingestion of large amounts of phenylalanine over a somewhat extended period might overwhelm the organism and, if homogentisic acid is an intermediary product of normal catabolism, result in alcaptonuria. In experiments of this type with white rats, it has been possible to produce alcaptonuria consistently provided that the amount of phenylalanine ingested was sufficiently great.

#### EXPERIMENTAL

Young and adult white rats were placed in individual metabolism cages, which permitted collection of the urine, and fed a basal diet, with which, during the experimental periods, varying percentages of the phenylalanine were incorporated. Except in a short series of experiments<sup>1</sup> with *dl*-phenylalanine (synthetic), the naturally occurring isomer, *l*(-)-phenylalanine,<sup>2</sup> was fed.

<sup>1</sup> The preliminary experiments were carried out by the senior author with the aid of Mr. Frank Koss.

<sup>2</sup> We wish to express our indebtedness to Dr. Seiichi Izume of Dairen, Manchuria, a former student in this laboratory, for the gift of a very considerable amount of *l*-phenylalanine which has made possible the conduct of these experiments.

This product contained small amounts of tyrosine as an impurity; the results obtained with it, however, were the same as those obtained when the *dl*-phenylalanine was fed. The basal diet contained the following substances: dried skim milk powder, 45 per cent; butter, 25 per cent; corn-starch, 27 per cent; cod liver oil, 3 per cent. Each animal received one tablet of dried yeast (approximately 400 mg.) daily. When phenylalanine was added to the diet, it replaced an equal weight of starch. The fat content of this diet was kept at a relatively high level in order to insure a consistency such that loss of the food by scattering would be reduced to a minimum. A careful record of the daily food intake was kept and the rats were weighed weekly. In the initial series, control experiments in which no phenylalanine was fed were carried out.

The urine, as it drained from large glass funnels in which the metabolism cages were placed, was filtered through a layer of coarse cheese-cloth to avoid contamination with particles of food or feces and was collected in large test-tubes containing a few drops of chloroform as a preservative and a layer of mineral oil to prevent contact with air and thus to diminish oxidation of the homogentisic acid. In some experiments, a few drops of 12 per cent sulfuric acid were placed in the collecting tubes, since it is known that oxidation of homogentisic acid occurs less readily in an acid solution.

For the qualitative detection of homogentisic acid in urine, the usual tests were employed; *i.e.*, brown-black pigmentation on addition of alkali and aeration (referred to in Table I and elsewhere as the alkali test), reduction of ammoniacal silver nitrate in the cold, and the production of a transitory blue color on the addition of a few drops of dilute ferric chloride. None of the methods for the quantitative determination of homogentisic acid is entirely satisfactory in experiments with small animals in which the volume of urine is scanty, as in the present series. We have made use of the method of Briggs (7), which requires only 1 to 2 cc. of urine. In some experiments, total phenols were determined by the method of Folin and Denis (8) with the use of the improved color reagent of Folin and Ciocalteu (9). Amino acid nitrogen was determined colorimetrically with the use of the naphthoquinone sulfonic acid reagent. Since homogentisic acid is a diphenol derivative and polyphenolic substances react with



the uric acid reagent of Folin and Denis, we have determined the chromogenic value of the urine with this reagent also. The preliminary treatment of the urine was the same as in the phenol method, but the color development was obtained with the uric acid reagent instead of the phenol reagent. The values obtained in this way are calculated in terms of the uric acid used as a standard and are designated as "polyphenols" in Table I. The polyphenol values are for comparative purposes only. An alcaptonuria should result in a marked rise in both the phenol and polyphenol content of the urine. It should also be noted that phenylpyruvic acid, a possible intermediate in the catabolism of phenylalanine, also reacts with the phenol reagent (10). The reactions and procedures for the isolation of homogentisic acid from rat urine will be discussed in detail later in this paper.

#### DISCUSSION

In the unpublished experiments carried out by one of us, previous to the present series, diets containing 4, 6, and 8 per cent of phenylalanine were fed to eighteen young white rats. Qualitative tests for homogentisic acid in the urine were obtained in thirteen of these animals, the period of preliminary feeding prior to the appearance of a positive test and the intensity of the test varying with the individual animal and with the daily intake of phenylalanine in the food. As a rule, qualitative tests were obtained in the urine if the phenylalanine of the diet exceeded 0.4 gm. per 100 gm. of rat, although no alcaptonuria was produced in one rat whose daily intake of phenylalanine averaged 0.54 gm. of phenylalanine per 100 gm. of body weight.

In the present series, with diets containing 6 to 12 per cent of *l*(-)-phenylalanine, experimental alcaptonuria was produced in sixteen of a total of seventeen rats, nine of which were adult animals. In the one exceptional case, the daily consumption of phenylalanine averaged less than 0.19 gm. per 100 gm. of rat for a period of 8 days. In no case were the tests for homogentisic acid obtained in the urine when the daily intake of phenylalanine was less than 0.3 gm. per 100 gm. The number of days required for the appearance of alcaptonuria after the animal received the experimental phenylalanine diet varied from 2 to 28 days. The reactions for homogentisic acid were absent from the urine 24

hours after the phenylalanine was removed from the diet. Unfortunately, we have no evidence as to the degree of absorption of phenylalanine in our experiments. That the amino acid absorbed was well utilized is indicated by the low values for amino acid nitrogen in the experimental urines.

A typical series of analytical data is presented in Table I. When the data for Rats 1 to 3 which received no phenylalanine are compared with the data for Rats 4 and 5, it will be seen that the administration of phenylalanine resulted in little change in the amino acid content of the urine. The phenol and polyphenol values were significantly increased whenever a strongly positive alkali test for homogentisic acid was obtained in the urine. It may be noted that it was impossible to check oxidation completely in the urines containing homogentisic acid and that occasionally, as indicated in Table I, the colors developed in the colorimetric procedures were unsatisfactory, particularly in the analyses of the pigmented urines.

All of the quantitative procedures and the qualitative tests obtained indicated that the rats ingesting large amounts of phenylalanine daily excreted homogentisic acid; that we had produced an experimental alcaptonuria by dietary factors. For final proof, however, isolation of a stable derivative of homogentisic acid from the experimental urines and its identification were necessary. We were fortunate in having available urine of two alcaptonuric brothers, patients in the University Hospital,<sup>3</sup> from which we were able to isolate large amounts of the insoluble lead salt of homogentisic acid (11) and to prepare pure homogentisic acid from the lead salt. This made possible preparation of derivatives of pure homogentisic acid for comparison with the derivatives isolated from the experimental rat urines.

Our first attempts at isolation by the precipitation of the lead homogentisate (11), either directly from the rat urines or from ether extracts of these urines, were unsuccessful. Small quantities of an impure compound were obtained, but the amount was too small for purification or characterization. Experiments with normal urine, to which small amounts of homogentisic acid were

<sup>3</sup> We are indebted to Dr. Moses Fröhlich of the Department of Medicine for cooperation which made possible the studies on these patients. These studies will be reported elsewhere.

TABLE I

*Composition of Urine of Rats Receiving Basal Control Diet and Basal Control Diet to Which 10 or 12 Per Cent L-Phenylalanine Has Been Added (Series II-A)*

The rats, which weighed from 100 to 115 gm., either gained slightly or remained approximately constant in weight. Food consumptions and urinary excretions are calculated on the basis of 24 hours. The phenylalanine was fed to the two experimental rats at a 10 per cent level for the first 7 days and at a 12 per cent level thereafter.

Rat No.	Sex	Period	Food intake			Urinary excretion				Alkali test
			Total	Phenylalanine		Phenols	Poly-phenols	Amino N	Homo-gen-tic acid	
				Total	Per 100 gm. rat					
		days	gm.	gm.	mg.	mg.	mg.	mg.	mg.	
1	F.	24*	6.6	0	0	8.7	1.9	4.7	0	—
2	M.	24*	6.2	0	0	9.4	1.7	3.6	0	—
3	"	24*	4.1	0	0	8.8	1.1	4.3	0	—
4	"	1-2	4.3	0.43	0.42	4.1	2.6	1.1	0	—
		3-4	4.0	0.40	0.40	6.9	2.0	2.3	0	—
		5-6	3.2	0.32	0.33	10.3	1.6	4.4	0	—
		7-8	3.2	0.38	0.40	10.9	2.2	4.0	0	—
		9-10	3.0	0.36	0.38	19.3	1.1	4.5	0	—?
		11-12	3.9	0.47	0.48	18.8	3.2	7.0	35.1	+
		13-14	4.6	0.55	0.56	13.7	3.0	2.1	4.4	+
		15-16	5.5	0.66	0.66	32.5	5.0	3.3	29.0	++
		17-18	5.0	0.60	0.58	50.0†	5.0‡	4.8	12.0	++
		19-20	6.5	0.78	0.73	104.0†	8.0‡		37.0	++
		21-22	6.9	0.83	0.74	137.0	18.0		29.0	++
		23-24	6.8	0.82	0.69	89.0	11.0		20.0	++
		5	F.	1-4	2.5	0.25	0.23	3.0	1.3	0.6
5-6	4.8			0.48	0.47	7.6	1.9	3.2	0.0	—
7-8	4.4			0.53	0.53	6.7	2.0	1.6	3.0	+
9-10	3.0			0.36	0.36	13.0	1.3	1.9	3.3	++
11-12	3.3			0.40	0.40	21.0	3.8	8.0	6.7	++
13-14	3.6			0.43	0.42	32.0	15.0	2.6	15.2	+
15-16	3.8			0.46	0.45	25.0	1.4	1.5	0.0	?
17-18	3.4			0.41	0.41	21.0	1.2	3.2	0.0	?
19-20	3.8			0.46	0.46	23.0†	9.5‡		7.5	++
21-22	4.1			0.49	0.48	101.0	19.0		36.0	++
23-24	4.8			0.58	0.56	70.0	45.0		45.0	++

\* For these control rats, the values are averages for the feeding period of 24 days.

† Phenol determination, purplish color, difficult to read accurately.

‡ Polyphenol determination, greenish color, difficult to read accurately.

added, convinced us that the method of the isolation of the lead salt could hardly be expected to be of value in urines containing amounts of homogentisic acid as small as those which were indicated by our quantitative analyses. Our efforts were then directed toward the isolation of the benzoyl derivatives described by Orton and Garrod (12). The melting point of dibenzoyl-homogentisamide has been reported to be  $204^{\circ}$  (12) and  $202^{\circ}$  (13). From the amide, the dibenzoylhomogentisic acid with a melting point of  $179-180^{\circ}$  (12) or  $180-181^{\circ}$  (14) may be obtained.

The mineral oil used to protect the rat urine from contact with air was removed and the urine was either benzoylated directly or after decolorization with norit. To 100 cc. of urine, 5 cc. of benzoyl chloride and 40 cc. of 10 per cent sodium hydroxide were added alternately in small portions, with vigorous shaking after each addition and cooling in an ice bath. When all the reagents had been added, the mixture was shaken until the odor of benzoyl chloride was no longer evident and allowed to stand in an ice bath for about 1 hour. The precipitate which separated was removed by centrifugation and extracted repeatedly with boiling ethyl alcohol in which the dibenzoylhomogentisamide is soluble. If necessary, the alcoholic extract was decolorized by norit. After the extracts had stood overnight in the refrigerator, the precipitate which separated was filtered off and dissolved in boiling alcohol. The solution was again decolorized and filtered rapidly by suction into a tube immersed in ice. Needle crystals separated. These were filtered off and, if necessary, the processes of solution, decolorization, and crystallization, were repeated. A total of approximately 1.2 gm. of crystals was thus isolated from three portions of experimental alcaptonuric urine, representing a total volume of 600 cc. The melting points ranged from  $189-200^{\circ}$ . These crystals were combined for further purification.

The combined crystals were thrice recrystallized from hot alcohol and the final product was compared with the dibenzoylhomogentisamide prepared from pure homogentisic acid (isolated from human alcaptonuric urine). The derivative from rat urine melted at  $199-200^{\circ}$ ; that from pure homogentisic acid at  $200-201^{\circ}$ ; and the melting point of a mixture of the two derivatives was  $200^{\circ}$  (all values are corrected). Both products gave the alkali test and showed the solubilities described by Orton and Garrod (12).

After the final recrystallization, approximately 160 mg. of the dibenzoylhomogentisamide were obtained from 600 cc. of rat urine.

Analysis of the two derivatives by the micromethods of Pregl<sup>4</sup> gave the following results: derivative from rat urine, C 70.31, H 4.56 per cent; derivative from pure homogentisic acid isolated from human alcaptonuric urine, C 70.30, H 4.48 per cent; theoretical for dibenzoylhomogentisamide, C 70.40, H 4.53 per cent.

As further proof of the identity of the derivative from rat urine and that from pure homogentisic acid, dibenzoylhomogentisic acid was prepared from each of the amides as described by Orton and Garrod (12). The melting points of the two products were 179–180° and 180–181° respectively (corrected) with a mixed melting point of 180–181°, values comparable to the values previously reported (12, 14). The dibenzoylhomogentisic acids showed identical properties, crystallized in tiny clusters of white needles, dissolved readily in 95 per cent alcohol at room temperature, crystallized from 50 per cent alcohol, and gave positive alkali tests.

Attempts to demonstrate similarly the production of alcaptonuria by feeding the sodium salt of phenylpyruvic acid, a probable product of catabolism of phenylalanine, were not successful. Large amounts of phenylpyruvic acid were excreted, as evidenced by the color reaction with ferric chloride, characteristic of phenylpyruvic acid (10), but no definitely positive alkali test could be obtained from the urine. The rats did not eat the diet containing phenylpyruvic acid well and persisted in scattering the food, so that the experiment was discontinued. The failure to obtain evidence of excretion of homogentisic acid by feeding the salt of phenylpyruvic acid may have been due to a lack of sufficiently high food intake and of a sufficiently long period of time. In view of the fact that phenylpyruvic acid increased the excretion of homogentisic acid, when fed to human alcaptonuric patients ((2) p. 79), it seems probable that our negative results with rats are thus to be explained.

We have also failed in attempts to produce experimental alcaptonuria in two rabbits by feeding phenylalanine. Phenylalanine, as the sodium salt, was fed daily to a 2 kilo rabbit by stomach

<sup>4</sup> The microanalytical determinations were carried out in the laboratory of Dr. Hans T. Clarke, to whom we wish to express our appreciation.

tube. On the first 2 days, 4 gm. daily were fed; on the next 6 days, 6 gm. daily; and on the 9th day, 8 gm. No alkali or transitory ferric chloride test was obtained with the urines, but all of the urines gave the intense grass-green coloration with ferric chloride, which is characteristic of phenylpyruvic acid. These findings are in confirmation of previous work from this laboratory (10).

The experimental evidence detailed here demonstrates conclusively the experimental production of alcaptonuria in normal animals. It should be pointed out that for consistent production of experimental alcaptonuria in our animals, a daily intake of at least 3.0 gm. of phenylalanine per kilo was necessary. On the basis of our data, it would seem that the amounts of amino acid fed in earlier experiments with other species, including man, would hardly have been sufficiently great to have given rise to the urinary excretion of homogentisic acid. Even though in our experiments the amounts of phenylalanine fed were large, far in excess of the content of phenylalanine in the normal diet of the rat, we feel that our findings lend support to the theory of Neubauer (3) and that homogentisic acid may be regarded as a normal product of intermediary metabolism.

#### SUMMARY

1. After the daily oral administration of *l*(-)-phenylalanine in amounts in excess of 0.3 gm. per 100 gm. of body weight to white rats, over a considerable period of time, the excretion of homogentisic acid in the urine was observed. The presence of homogentisic acid was demonstrated, not only by qualitative and quantitative tests, but also by the preparation, from the experimental urines, of a derivative of homogentisic acid, dibenzoylhomogentisamide, which was characterized by its melting point and elementary analysis.

2. These results lend support to the theory that homogentisic acid is an intermediate product of the normal metabolism of phenylalanine.

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## THE AVAILABILITY OF CALCIUM FROM CHINESE CABBAGE (*BRASSICA PEKINENSIS*, RUPR.)

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McClugage and Mendel (1) reported that the calcium of spinach and carrots was poorly utilized by dogs, and suggested that these green vegetables should not be extensively used as a dietary source of calcium in place of milk. Rose (2), however, obtained positive calcium balances on women subjects receiving a diet in which more than half of the dietary calcium was supplied by carrots. Blatherwick and Long (3) concluded that the calcium derived from vegetables was capable of meeting the maintenance needs of men.

Rose and MacLeod (4) found that when almonds furnished 73 per cent of the calcium of the diet, the calcium was as well utilized as that of milk or carrots. When, however, the almonds contributed 85 to 86 per cent of calcium, the utilization was not as efficient. Sherman and Hawley (5) found as a result of studies conducted on growing children that for these subjects the utilization of the calcium of vegetables was not as favorable as that of milk. Mallon, Johnson, and Darby (6) concluded, as a result of studies conducted on adult women, that the calcium of green lettuce was as well utilized as that of milk. Adolph and Chen (7) also reported that the calcium of soy bean curd cheese was as well utilized as that of milk. Fincke and Sherman (8) found that the calcium of kale was nearly as well utilized as the calcium of milk, but the calcium of spinach was utilized poorly if at all. Recently, Horwitt, Cowgill, and Mendel (9) upon conducting *in vitro* determinations found that about 30 per cent of the calcium of spinach was rendered soluble under the conditions of their experiment.



*Plan of Present Work*

Whereas in the western hemisphere cow's milk generally serves as an excellent source of calcium, in some Oriental countries it is rarely used, owing to its high cost and difficulty of adaptation to the traditional dietary habits of the population. The usual Chinese dietary consists largely, if not entirely, of cereals supplemented with green vegetables. As cereals are known to be inadequate in their calcium content, the only adequate known source of this element would be from the vegetables which supplement them in the dietary. Therefore this investigation was undertaken to determine the availability of the calcium of one of these typical and widely used vegetables; namely, the Chinese cabbage (*Brassica pekinensis*, Rupr.).

Young healthy albino rats which had been reared to 4 weeks of age on Diet 13 of this laboratory (one-third whole cow's milk powder, two-thirds ground whole wheat with sodium chloride added to the extent of 2 per cent of the weight of the wheat) were placed on diets closely resembling Diet 13. In these experiments the milk was used in the form of skim milk powder, the butter fat being added separately. In the diet with which it was compared, half of the skim milk powder was replaced by sufficient Chinese cabbage to supply an equivalent amount of calcium. The fiber content of the two diets was also adjusted by adding Chinese cabbage fiber to the milk diet. At 60 days of age the animals were killed, their bodies analyzed for calcium, and the storage calculated.

## EXPERIMENTAL AND DISCUSSION

The Chinese cabbage was bought from time to time during the winter of 1936 to 1937 in an open market in New York. The rib leaf parts were separated from the stems and roots, thoroughly washed with tap water, rinsed with distilled water, and air-dried at room temperature and finally in an electric oven at 70° to avoid charring of the leaves. It was found that during this process of drying about 95 per cent of the fresh weight of the leaves was lost as moisture. The dried leaves were then finely ground and the whole amount mixed thoroughly together and finally stored in stoppered bottles. Two lots of the cabbage were prepared, the first lot containing more of the inner white leaves

and the second lot more of the outer green leaves. After the above method of drying, the two lots were thoroughly mixed together and sampled for analysis with the results shown in Table I.

The same lot of skim milk powder was used throughout the entire investigation. The skim milk powder was analyzed with the results shown in Table II.

Calcium was determined by the modified McCrudden method as described in previous publications (10) from this laboratory, phosphorus by the double precipitation method of the Association of

TABLE I

*Protein, Fat, Crude Fiber, Calcium, Phosphorus, and Moisture Contents of Dried Chinese Cabbage*

	per cent
Protein ( $N \times 6.25$ ).....	19.55
Fat (ether extraction for 24 hrs.).....	2.12
Crude fiber.....	10.62
Calcium.....	0.84
Phosphorus.....	0.74
Moisture.....	2.09

TABLE II

*Calcium, Phosphorus, and Protein Contents of Skim Milk Powder*

	per cent
Calcium.....	1.28
Phosphorus.....	1.05
Protein ( $N \times 6.25$ )...	33.07

Official Agricultural Chemists, and nitrogen by the usual Kjeldahl method with copper sulfate as a catalyst.

The butter fat used in this investigation was prepared from whole butter purchased from time to time in the open market. The whole butter was melted at about 65°, filtered, and the butter fat thus obtained stored under refrigeration until being used. The Chinese cabbage fiber was prepared from the dried leaves according to the method of the Association of Official Agricultural Chemists for crude fiber determinations, without the addition of asbestos. The dried fiber was ground to a fine powder and upon

analysis was found to have an ash content of 0.67 per cent and a calcium content of 0.08 per cent.

The composition and analysis of the experimental diets are given in Table III. The diets were so planned that they contained approximately the same amount of calcium, phosphorus, and protein.

Young, healthy, albino rats 28 days old, which had been reared on Diet 13, were placed in individual cages with raised screen bottoms and continued on the experimental diets until 60 days of age. Litter mates of the same sex were compared. The animals

TABLE III  
*Composition of Diets Containing Equivalent Amounts of Calcium*

	Diet 771, control	Diet 772, Chinese cabbage
	<i>per cent</i>	<i>per cent</i>
Whole wheat .....	64.0	64.0
Skim milk powder .....	21.8	10.9
Butter fat .....	6.9	6.9
Dried Chinese cabbage .....		16.9
Chinese cabbage fiber .....	1.8	
Corn-starch .....	4.2	
NaCl .....	1.3	1.3
	100.0	100.0
Calcium .....	0.31	0.31
Phosphorus .....	0.42	0.42
Protein (N $\times$ 6.25) .....	14.44	14.18

were so chosen that the initial body weights of the groups to be compared were approximately the same. Food and distilled water were available *ad libitum*. Records were kept of the amounts of food eaten, any food which was spilled being carefully separated from the feces and returned to the food cup. All the animals grew normally and were apparently in good health. However, the average growth of the animals on the control diet was slightly higher than the average growth of those on the cabbage diet, as shown in Table IV.

At 60 days of age the rats were weighed, killed with chloroform, and brushed to remove any food particles from the fur. The

gastrointestinal tract was dissected out, the contents removed, and the weight of the contents subtracted from the final weight of the rat to give net weight which was used as a basis in all calculations. The alimentary tract was discarded, as it had been previously shown in this laboratory that the calcium in the walls of the intestinal tract is negligible. The rats were then charred

TABLE IV

*Average Growth of Rats from 28th to 60th Day on Diets 771 and 772*

Diet No.	Sex (16 rats in each experiment)	Average initial body weight	Average net gain in body weight	Average total food intake	Average gain per gm. food	Average gain per gm. protein
		gm.	gm.	gm.	gm.	gm.
771	M.	52±1*	74±2*	240	0.31±0.005*	2.13±0.04*
772	"	51±1	50±2	214	0.23±0.005	1.64±0.03
771	F.	44±1	60±2	221	0.27±0.004	1.85±0.03
772	"	44±1	45±2	205	0.22±0.003	1.53±0.03

\* ± average deviation of the mean.

TABLE V

*Calcium Content of 60 Day-Old Rats Fed on Diets Containing Same Amount of Calcium from Milk or Chinese Cabbage*

Diet No.	Sex (16 rats in each experiment)	Average net body weight	Average Ca intake	Average total body Ca	Per cent body Ca	Ca utilization factor
		gm.	gm.	gm.		
771	M.	121±3*	0.739±0.013*	0.994±0.016*	0.83±0.008*	0.88±0.006*
772	"	97±2	0.656±0.019	0.870±0.017	0.90±0.005	0.80±0.008
771	F.	100±2	0.681±0.015	0.900±0.015	0.90±0.005	0.88±0.007
772	"	85±2	0.631±0.016	0.792±0.013	0.93±0.004	0.78±0.010

: average deviation of the mean.

and ashed according to the usual technique of this laboratory. The ash was dissolved in hydrochloric acid, filtered, diluted to 500 cc. in a volumetric flask, and stored in glass-stoppered bottles. Aliquots of this solution were then taken for the calcium determinations according to the modified McCrudden method.

The results are summarized in Table V. The bodies of males on the control diet contained an average of  $0.83 \pm 0.008$  per cent

calcium; on the cabbage diet  $0.90 \pm 0.005$  per cent. The bodies of females contained averages of  $0.90 \pm 0.005$  per cent calcium on the control diet and  $0.93 \pm 0.004$  per cent calcium on the cabbage diet. Therefore the percentage of body calcium of both the male and female animals was slightly higher on the cabbage than on the control diet. This, however, is to be expected, as the animals on the control diet grew at a more rapid rate, the growth per gm. of food eaten being greater on the control than on the cabbage diet.

In order to obtain a more significant basis of comparison between the two diets, the calcium utilization factor, in Table V, was calculated. This was obtained by dividing the weight of calcium stored in the body of the animals which were on the experimental diets by the weight of calcium ingested in the food eaten during the same period. The amount of calcium stored was found by subtracting from the total amount of calcium in the body at 60 days, the amount of calcium in the body at 28 days. The calcium content of 60 day-old rats was determined directly as shown in Table V, while that of 28 day-old rats was determined by the analysis at 28 days of age of litter mates of the animals reared to 60 days of age on the control and cabbage diets. The body weight of these animals was therefore approximately the same at 28 days of age as that of the animals used in the experimental diets. The method followed for determining the calcium content of these animals was exactly the same as that previously described for the experimental animals. The results are given in Table VI. The average percentage of body calcium for the male rats at 28 days of age was  $0.74 \pm 0.008$  per cent and for the female rats was  $0.74 \pm 0.004$  per cent. These values compare favorably with that of 0.67 per cent for males and 0.74 per cent for females obtained previously in this laboratory by Sherman and MacLeod (10) and by Sherman and Booher (11) on comparable animals of the same age.

From Table V, it will be seen that the calcium utilization factor for the male animals on the control diet was 0.88 as compared with 0.80 for the males on the cabbage diet. In the case of the female animals, the calcium utilization factor was 0.88 on the control diet as compared with 0.78 on the cabbage diet.

It is apparent from these values that sexual differences did

not influence the calcium utilization factor on either the control or cabbage diet, since the average utilization factors for the male and female animals were identical on the control diet, and on the cabbage diet the difference was only of the order of 0.02 with a probable error of the difference of 0.012.

Table VII contains the probable error and coefficient of variation obtained of the calcium utilization factors given in Table V.

TABLE VI  
*Calcium Content of 28 Day-Old Rats Reared on Diet 13*

Sex (16 rats in each experiment)	Average body weight	Average net body weight	Average total body Ca	Per cent body Ca
	gm.	gm.	gm.	
M.	52±2*	47±2*	0.345±0.008*	0.74±0.008*
F.	45±1	42±1	0.309±0.005	0.74±0.004

\* ± average deviation of the mean.

TABLE VII  
*Average Calcium Utilization Factors of Control and Cabbage Diets with Their Probable Error and Coefficient of Variation*

Diet	Sex	Average Ca utilization factor	Probable error	Coefficient of variation*
771. Control.....	M.	0.88	±0.005	3.6
772. Chinese cabbage.....	"	0.80	±0.007	4.9
771. Control.....	F.	0.88	±0.007	4.4
772. Chinese cabbage.....	"	0.78	±0.009	6.7

\* Coefficient of variation of individual calcium utilization factors.

From Table VII it is apparent that in comparing the control diet with the cabbage diet, the difference between the utilization factors was 0.08 for the males with a probable error of the difference of 0.009, and 0.10 for the females with a probable error of the difference of 0.012. These differences are respectively 9 times and 8 times their probable errors, and therefore are significant. Hence it is apparent that the calcium of Chinese cabbage is almost but not quite as well utilized as that of milk, the utilization on the Chinese cabbage diet being approximately nine-tenths of that obtained on the milk diet.

## SUMMARY

In these experiments young, healthy, albino rats of the same nutritional history were placed, when 4 weeks old, on a diet in which practically all of the calcium was supplied by skim milk or on a diet in which half of the skim milk was replaced by enough finely ground dried Chinese cabbage (*Brassica pekinensis*, Rupr.) to provide the same amount of calcium. At 60 days of age the animals were killed and their bodies analyzed for calcium.

It was found that the calcium of Chinese cabbage was almost as well utilized as that of milk, the utilization on the Chinese cabbage diet being approximately nine-tenths of that obtained on the milk diet.

It would therefore appear that Chinese cabbage probably serves as an excellent and available source of calcium in the Chinese dietary.

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## **STUDIES ON BIOLOGICAL OXIDATIONS**

### **X. THE OXIDATION OF UNSATURATED FATTY ACIDS WITH BLOOD HEMIN AND HEMOCHROMOGENS AS CATALYSTS**

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The catalytic power of heme compounds (blood hemin and hemoglobin) on the oxidation of unsaturated fatty acids, first discovered by Robinson (1) and studied afterwards by a number of investigators, is of biological importance because of the widespread presence of hemes and hemochromogens in living cells. This catalytic power was reported to be insensitive to the inhibiting power of HCN. In many of the experiments no distinction was made between the catalytic power of hemin and that of hemochromogens, for in most cases pyridine (a base which forms a hemochromogen) was used to dissolve hemin. In experiments on the effect of HCN fundamental precautions were disregarded. For these reasons a reinvestigation of the problem was considered necessary. In this paper are presented data on the catalytic power of hemin and hemochromogens on the oxidation of linseed oil and oleic acid, the effect of HCN on this catalytic power, and the effect of substances usually considered inhibitors of chain reactions. A study of these experiments favors the view that the oxidation of unsaturated fatty acids with hemes as catalysts is of a chain reaction mechanism.

#### **EXPERIMENTAL**

The rate of oxidation was determined with the Warburg-Barcroft microrespiration apparatus. The pH values of the solutions were adjusted in each experiment by electrometric titration with the glass electrode. To avoid uncontrollable influences, in all these experiments, one sample of linseed oil and one of oleic acid



were used. In the absence of a catalyst these samples were not oxidized by atmospheric oxygen in 4 hours at 37° when in suspension in buffer solutions of the pH values used in these experiments.

*Effect of pH on Oxidation of Linseed Oil with Blood Hemin As Catalyst*—The rate of oxidation of linseed oil with ferricyanide as catalyst increases with the hydroxyl ion concentration, according to Chow (2). Kuhn and Meyer (3), studying the catalytic power of hemin on the oxidation of oleic acid, concluded that the optimum pH for this oxidation was between 7 and 8. (Their experiments were made only up to pH 9.05, and the precipitation of hemin starts at pH 6.6.) In the experiments reported here the rate of oxidation of linseed oil with hemin as catalyst increased with the hydrogen ion concentration (Table I). This increase may be attributed to the effect of pH on the oxidation-reduction

TABLE I

*Effect of pH on Oxidation of Linseed Oil by Atmospheric Oxygen with Hemin As Catalyst*

Concentration of linseed oil, 3 per cent; concentration of hemin,  $8 \times 10^{-5}$  mole per liter. Temperature, 38°.

pH.....	6.96	8.10	9.19	9.93	11.88
O <sub>2</sub> uptake in 30 min., c.mm. ....	542	503	475	363	260

potential of blood hemin, since the potential becomes 60 millivolts more positive for a change of 1 pH unit (Barron (4)).

*Catalytic Power of Blood Hemin and Hemochromogens on Oxidation of Linseed Oil by Atmospheric Oxygen*—These experiments were performed in order to compare the rate of reaction to the free energy of the catalysts, the oxidation-reduction potentials of which were determined by Barron (4). Unfortunately the large amount of base used to prevent dissociation of the hemochromogen altered to varying degrees the state of aggregation of the fatty acid molecule; furthermore many of these bases acted as inhibitors (nicotine at pH 12.01 inhibited the oxidation almost completely). This catalytic power was studied at pH 12.10 and 9.50. The catalytic powers of pilocarpine and histidine hemochromogens were the highest of all the hemochromogens studied (Table II). A similar finding on the high catalytic power of

imidazole hemochromogens was reported by Langenbeck, Hut-schenreuter, and Rottig (5). No relation was found between the oxidation-reduction potential of the catalyst and the rate of oxidation of linseed oil. It may be recalled that Chow and Kamerling (6) and Chow (2) suggested that the rate of oxidation of oleic acid catalyzed by ferricyanide, and the oxidation of linseed oil catalyzed by ferricyanide, tungsticyanide, and molybdicyanide depended on the oxidation-reduction potential of the catalysts. Although Franke (7) has reported that linoleic acid is oxidized by certain amino acids, among them histidine, linseed oil in our ex-

TABLE II

*Catalytic Power of Blood Hemin and Hemochromogens on Oxidation of Linseed Oil by Atmospheric Oxygen*

Concentration of linseed oil, 3 per cent; concentration of hemin,  $8 \times 10^{-5}$  mole per liter. Temperature, 38°.

Base	Concentration of base  mole per l.	O <sub>2</sub> uptake in 60 min.	
		pH 9.50	pH 12.10
Pilocarpine . . . . .	0.06	864	357
Histidine . . . . .	0.06	724	-
None . . . . .		591	106
Nicotine . . . . .	0.05	524	
Pyridine . . . . .	1.00	513	140
$\alpha$ -Picoline . . . . .	1.00	122	
Cyanide . . . . .	0.06	26	143

periments was not oxidized by histidine nor by any of the bases used for hemochromogen formation.

*Effect of HCN on Oxidation of Linseed Oil with Hemin and Hemochromogens As Catalysts*—The insensitivity to HCN of the catalytic power of heme compounds on the oxidation of fatty acids, first found by Robinson (1), has been the subject of repeated investigations. Unfortunately all these experiments were performed without due regard to (1) the degree of dissociation of HCN, (2) the possibility of formation of cyanide hemochromogen, (3) the rapid absorption of HCN by the KOH solutions used in the Warburg-Barcroft microrespirometers to absorb the CO<sub>2</sub> produced. Robinson did not give the pH of her solutions;

Wright and Van Alstyne (8) performed their experiments in 1 per cent  $\text{Na}_2\text{CO}_3$  solutions. Obviously they were comparing hemin and cyanide hemochromogen catalysis, and not the effect of HCN (pK of HCN, 9.1, Clark (9)). Kuhn and Meyer (3) in their experiments gave as their first figures on the rate of oxygen consumption those obtained 9 hours after addition of HCN. From Schmitt and Schmitt's measurements (10) on the rate of HCN absorption by KOH in Warburg vessels it is easily inferred

TABLE III

*Effect of HCN on Oxidation of Linseed Oil with Hemin and Cyanide Hemochromogen As Catalyst*

Concentration of hemin,  $8 \times 10^{-6}$  mole per liter.

pH		KCN, total concentration	HCN, con- centration	O <sub>2</sub> uptake in 30 min.	Inhibition
		<i>mole per l.</i>	<i>mole per l.</i>	<i>c.mm.</i>	<i>per cent</i>
Borate.	9.50			367	
"	9.50	0.06	0.0185	12.1	97
"	9.19			504	
"	9.19	0.06	0.0283	39.8	92
"	8.00			569	
"	8.00	0.05	0.046	204	64
Phosphate.	12.01			106	
"	12.01	0.1		172	Increase
"	8.00			629	
"	8.00	0.05	0.046	264	58
"	6.96			633	
"	6.96	0.05	0.05	643	None

that after 9 hours there was left in the solution a vanishingly small fraction of the added HCN.

The effect of HCN on the oxidation of linseed oil with hemin as catalyst was studied at different pH values and different HCN concentrations, the concentration of hemin being kept constant (Table III). All these experiments were of short duration (30 minutes) and the KOH solution used to absorb the  $\text{CO}_2$  produced was saturated with KCN to avoid distillation of HCN (Krebs (11)). For the interpretation of these experiments, the following must be taken into consideration: (1) the combination of hemin with  $\text{CN}^-$  to form cyanide hemochromogen, (2) the

dissociation constant of this hemochromogen, and (3) the dissociation constant of HCN. In borate buffer at pH 9.50 and 9.19, where the ratio  $\text{CN}^-:\text{HCN}$  was 2.24 and 1.12 respectively, HCN inhibited the oxidation of linseed oil to the extent of 97 and 92 per cent respectively. In both cases hemin was present largely as cyanide hemochromogen. At pH 8, when the ratio of  $\text{CN}^-:\text{HCN}$  was reduced to 0.087, thus diminishing the concentration of cyanide hemochromogen, the inhibition was reduced to 64 per cent. In phosphate buffer, at pH 12.01, where all the KCN added was practically in the form of  $\text{CN}^-$ , the rate of oxidation was greater than that produced by hemin alone. At pH 8, HCN caused an inhibition of 58 per cent. At pH 6.96, where there was practically no  $\text{CN}^-$  and therefore no hemochromogen formation, HCN had no effect on the oxidation of linseed oil with hemin as catalyst. Since this inhibition was higher at a hydrogen ion concentration where  $\text{CN}^-$  and HCN were present roughly in equal concentration, and disappeared as soon as either  $\text{CN}^-$  (pH 6.96) or HCN (pH 12.01) concentrations became vanishingly small, the inevitable conclusion was that HCN inhibited the catalytic power of cyanide hemochromogen but had no effect on hemin catalysis. This inhibiting effect of HCN on hemochromogen catalysis and not on hemin catalysis was also observed with pyridine hemochromogen at pH 7.01. In the presence of 2 M pyridine per liter (sufficient to maintain all the hemin as hemochromogen) 0.01 M per liter of HCN inhibited the oxidation completely. When the concentration of pyridine was lowered to 1 M per liter to allow dissociation of the hemochromogen with consequent formation of hemin, the inhibition was considerably reduced (Fig. 1).

*Effect of Chain Reaction Inhibitors on Oxidation of Linseed Oil—*

The autocatalytic character of the so called autoxidation of fats and fatty acids, the inhibition of this oxidation by addition of antioxidants, the inhibition of linseed oil oxidation with ferricyanide as catalyst on addition of a series of derivatives of aniline and phenol (Chow (2)), and the inhibiting effect of a number of phenol derivatives (Franke (7)) indicate that the oxidation of fatty acids might be of a chain reaction mechanism. The effect of inhibitors usually classed as chain reaction inhibitors on the oxidation of linseed oil and oleic acid with hemin as catalyst is

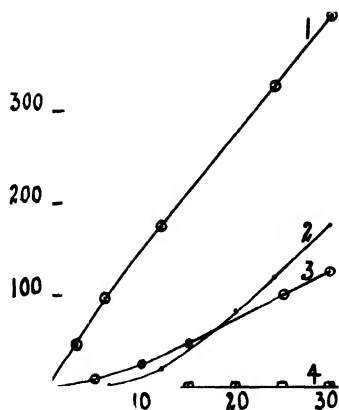


FIG. 1. Effect of HCN on the oxidation of linseed oil with pyridine hemochromogen as catalyst. pH, 7.01. Curve 1, pyridine hemochromogen with pyridine, 1 mole per liter; Curve 2, same with HCN, 0.01 mole per liter; Curve 3, pyridine hemochromogen with pyridine, 2 moles per liter; Curve 4, same with HCN, 0.01 mole per liter. Abscissa, time in minutes; ordinate,  $O_2$  uptake in c.mm.

TABLE IV

*Oxidation of Linseed Oil and Oleic Acid with Hemin As Catalyst. Effect of Chain Reaction Inhibitors*

The linseed oil oxidation was carried out at pH 6.96, temperature  $27^\circ$ , in air; hemin concentration,  $8 \times 10^{-5}$  mole per liter. The oleic acid oxidation, at pH 6.70, temperature  $38^\circ$ , in oxygen; hemin concentration  $1.6 \times 10^{-4}$  mole per liter.

Inhibitor	Concentration	Linseed oil inhibition	Oleic acid inhibition
	<i>mole per l.</i>	<i>per cent</i>	<i>per cent</i>
$\alpha$ -Naphthol.....	0.001	Complete	Complete
<i>p</i> -Aminophenol.....	0.001	95	
Catechol.....	0.001	94	85
Pyrogallol.....	0.001	91	52
2,6-Dichlorophenol indophenol.	0.00003	36	47
Dimethyl aniline.....	0.001 (About)	35	92
Resorcinol.....	0.001	22	54
<i>p</i> -Bromophenol.....	0.001	None	40

reported in Table IV. The oxidation of linseed oil was inhibited at varying degrees by all of them except *p*-bromophenol. The oxidation of oleic acid was inhibited by all the inhibitors used.

*Mechanism of Catalytic Effect of Hemin and Hemochromogens on Oxidation of Unsaturated Fatty Acids*—In the series of oxidation reactions studied in this laboratory, where heme compounds acted as catalysts (the oxidation of lactate activated by the activating enzyme of  $\alpha$ -hydroxyoxidase (12), the oxidation of ascorbic acid

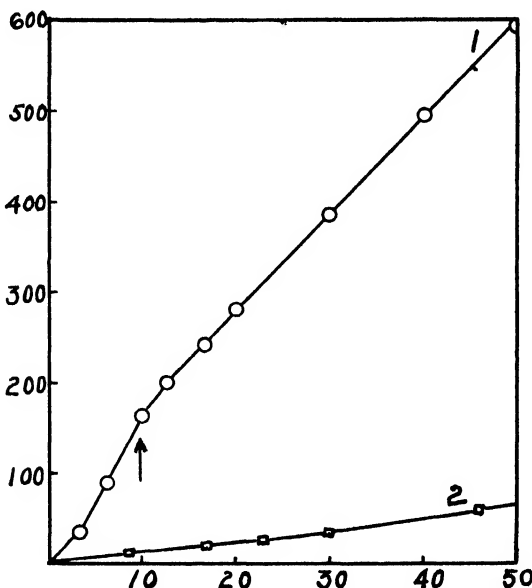
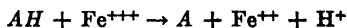


FIG. 2. Effect of HCN on the oxidation of linseed oil with cyanide hemochromogen as catalyst. pH, 9.19; temperature, 38°. Concentration of HCN, 0.0283 mole per liter; concentration of cyanide hemochromogen,  $8 \times 10^{-5}$  mole per liter. Abscissa, time in minutes; ordinate, O<sub>2</sub> uptake in c.mm. Curve 1, HCN added 10 minutes after hemin addition; Curve 2, HCN added before hemin addition.

(13), the oxidation of glutathione (14)) it has been shown that the reaction mechanism was of the general type



(where  $AH$  represents the oxidizable substrate and  $A$  the oxidation product), the ferro compound being reoxidized by atmospheric oxygen. When either linseed oil or oleic acid was mixed with a ferrihemochromogen in the absence of oxygen, the spectrum of ferrohemochromogen appeared very slowly and the concentration

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of it never exceeded 8 to 10 per cent of the ferrihemochromogen added. Similar findings were made by Wright, Connant, and Kamerling (15) when studying the catalytic effect of ferricyanide in the oxidation of oleic acid. It seems likely that the function of

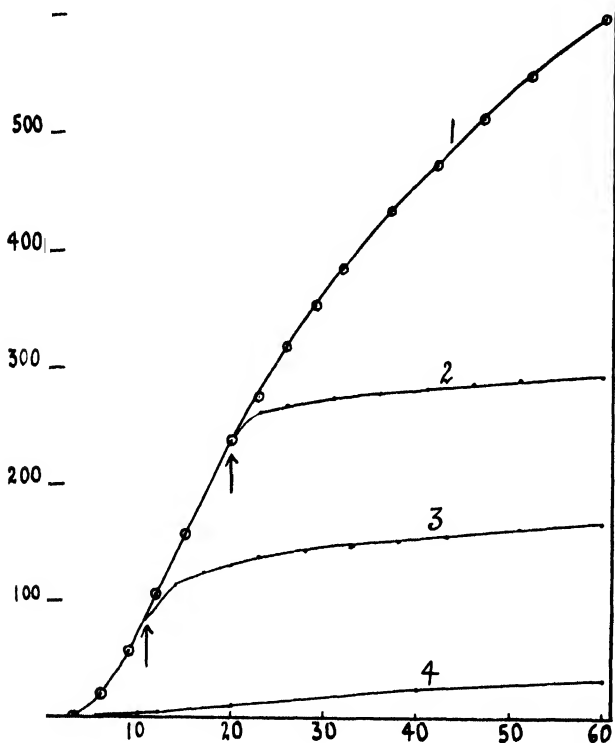


FIG. 3. Effect of *p*-aminophenol (0.001 mole per liter) on the oxidation of linseed oil with hemin as catalyst. pH, 6.96; temperature, 28°. Abscissa, time in minutes; ordinate, O<sub>2</sub> uptake in c.mm. Curve 1, control; Curve 2, *p*-aminophenol added 20 minutes after hemin addition; Curve 3, *p*-aminophenol added 10 minutes after hemin addition; Curve 4, *p*-aminophenol added before hemin addition.

heme compounds is similar to that of Cu<sup>++</sup> ions in the oxidation of sodium sulfite, which, according to Haber (16), have the function of facilitating the formation of initial centers responsible for the development of the chain. Once the chain reaction is started

and the reaction velocity has reached its constant value, the inhibiting effect must be quite small compared with its action when added at the initial period. This is clearly shown in experiments with HCN as the catalyst inhibitor and cyanide hemochromogen as the oxidizing catalyst (Fig. 2). HCN added before addition of the catalyst produced a large and permanent inhibition (92 per cent), but when it was added after the reaction velocity became constant the inhibiting power was small indeed. On the other hand, a chain reaction inhibitor will inhibit the reaction velocity of a chain reaction whether it is added at the start of the reaction or later, the inhibiting effect depending on the length of the chain and concentration of inhibitor. *p*-Aminophenol, a chain reaction inhibitor, was added either before or after addition of the catalyst. In every instance the inhibiting effect reached identical values (Fig. 3).

#### SUMMARY

The oxidation of linseed oil by atmospheric oxygen with hemin as catalyst increases with the hydrogen ion concentration up to pH 6.96. In a study of the reaction velocity of this catalysis with blood hemin and a series of hemochromogens, histidine and pilocarpine hemochromogens were found to be the most effective catalysts. HCN had no influence on the catalytic action of hemin but inhibited the catalytic action of hemochromogens. Chain reaction inhibitors inhibited the oxidation of linseed oil and oleic acid with hemin as catalyst. This action, together with a comparative study of the effect of catalyst inhibitors and chain reaction inhibitors, indicates that the oxidation of these fatty acids is of a chain reaction mechanism.

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# THE EFFECT OF JAUNDICED BLOOD UPON NORMAL DOGS, WITH SPECIAL REFERENCE TO THE SERUM PHOSPHATASE

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The origin of the increased serum phosphatase which occurs in certain diseases (1-4) is debated and uncertain. Its rate of production and removal from the blood stream is also unknown, as well as its influence upon other blood constituents. As a part of a study of this enzyme, we have determined the rate of removal of abnormal concentrations of phosphatase from the circulation of normal dogs. This study has also included, since the source of the enzyme was blood from jaundiced dogs, observations on the effect of jaundiced blood upon the serum inorganic phosphorus and sugar of normal dogs. Finally, the effect of the transfusion of normal blood upon normal recipients has been determined.

## *Methods*

The donors were all large (40 pounds or more) dogs whose common bile ducts had been ligated, with the exception of the normal donors, 10 to 15 days prior to the time of transfusion. Usually one donor supplied sufficient blood for two recipients. The recipients were all medium sized (approximately 25 pounds) dogs that had been fasted for 12 hours before the transfusion.

Every experiment was preceded by cross-agglutination tests and no incompatible recipients were used. The donor and both recipients were anesthetized with nembutal (1 grain for every 5 pounds) a few minutes before the transfusion; then the common carotid of the donor was exposed and prepared for cannulation; next the external jugular vein of each recipient was exposed and

\* China Foundation Fellow.

prepared for cannulation. A 300 cc. glass bulb<sup>1</sup> with one end drawn into a cannular form was used to transport blood from the donor to the recipient. The glass bulb was coated with paraffin and no anticoagulant was used. 250 to 300 cc. of blood were transfused, according to the weight of the recipient. The transfusions were given rapidly and both were completed within 5 minutes. Samples of blood were drawn from the donor and both recipients just before the transfusion and from the recipients 5 minutes, 1 hour, 2 hours, 4 hours, and 6 hours after the transfu-

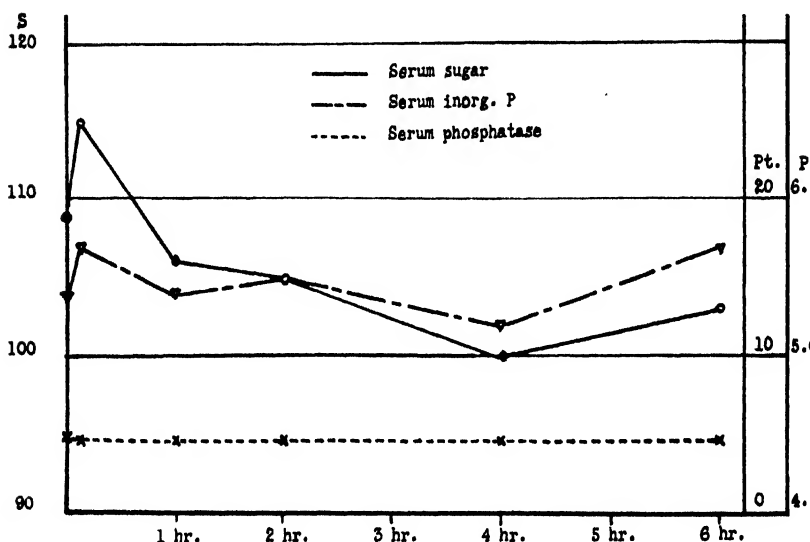


FIG. 1. Average serum values on ten dogs given transfusions of normal blood.

sion; some of the recipients were studied for several days following the transfusion. The following analytical methods were employed: serum inorganic phosphorus and phosphatase according to Bodansky (5), and the serum sugar after Shaffer and Hartmann as modified by Somogyi (6), with the latter's method (7) of preparing a protein-free filtrate of serum.

<sup>1</sup> We are indebted to Dr. R. B. Lewis of the Chemistry Department for making these bulbs.

## EXPERIMENTAL

*Transfusion with Normal Blood*—Fig. 1 shows the average results of ten dogs given transfusions of normal blood. It shows that such a transfusion has no effect upon the serum phosphatase;

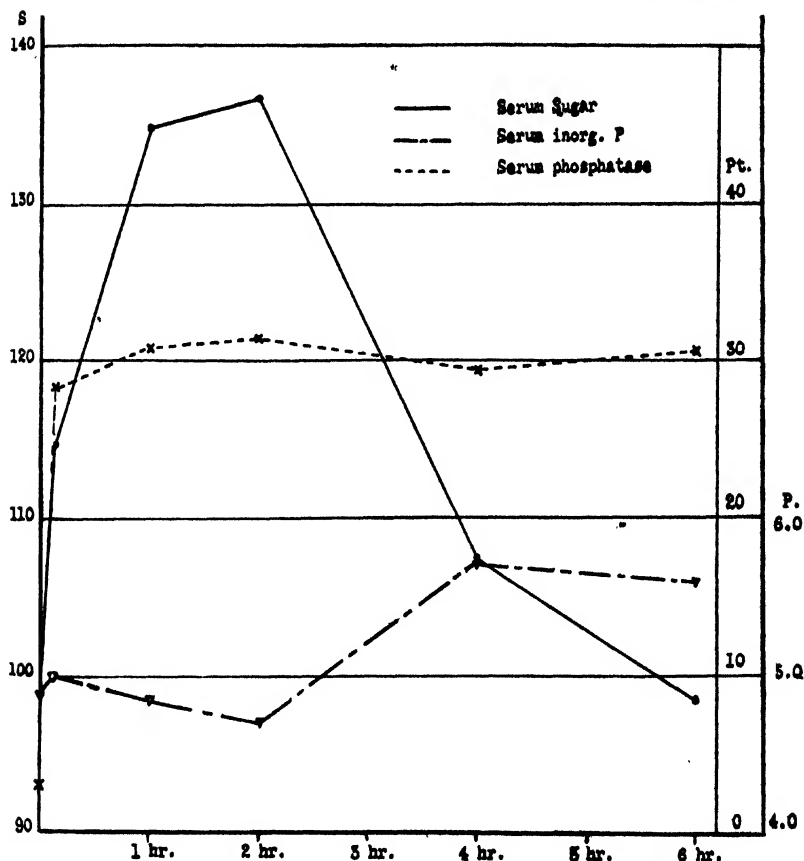


FIG. 2. Average serum values for ten dogs showing an increase in serum sugar after receiving jaundiced blood.

a transient rise in blood sugar occurs with a return to the pre-transfusion level by the end of the 1st hour. The changes in serum inorganic phosphorus are irregular and inconstant.

*Transfusion with Jaundiced Blood*—Fifteen recipients were given transfusions of jaundiced blood. Ten showed a serum

sugar rise of 10 mg. or more and the others showed either no elevation or a decline in serum sugar after the transfusion. The results have been divided on the basis of the serum sugar response. The average serum values of the hyperglycemic group are presented in Fig. 2; those for the other five dogs in Fig. 3. Fig. 2 shows a hyperglycemia which begins promptly after the transfusion, reaches a maximum between the 1st and 2nd hours, and subsides to approximately normal by the end of the 4th hour. The serum inorganic phosphorus in some instances appears to

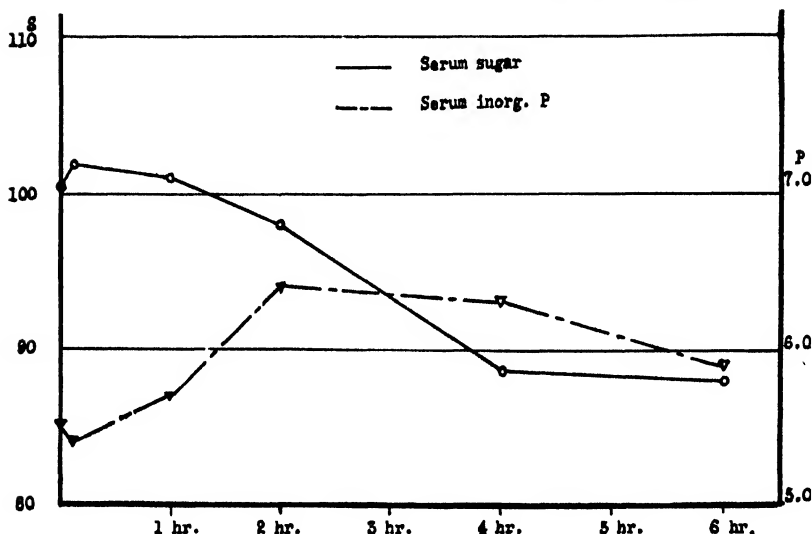


FIG. 3. Average serum values for five dogs given transfusions of jaundiced blood, but showing no increase in serum sugar.

bear an inverse relation to the serum sugar, yet the predominant tendency is for the serum phosphorus eventually to rise, usually by the end of the 4th or 6th hour. The serum phosphatase invariably rose to a high level and remained unchanged throughout the course of the experiment. Fig. 3 shows the results on five of the fifteen dogs receiving jaundiced blood. The serum sugar showed practically no rise in any instance (less than 10 mg.), and four of the dogs showed a definite decrease in 4 or 6 hours. The serum inorganic phosphorus rose in every instance, the maximum rise generally occurring 2 or 4 hours after the transfu-

sion. The behavior of the serum phosphatase in this group is not included, as it was identical with that shown in Fig. 2.

The return of the serum phosphatase toward normal is shown in Fig. 4 and one notes that the rate of removal is much more rapid for high concentrations of the enzyme in the serum, and that the increased concentration is only slowly reduced as the level approaches normal; it has not completely returned to the pre-transfusion level as late as the 8th day after the transfusion.

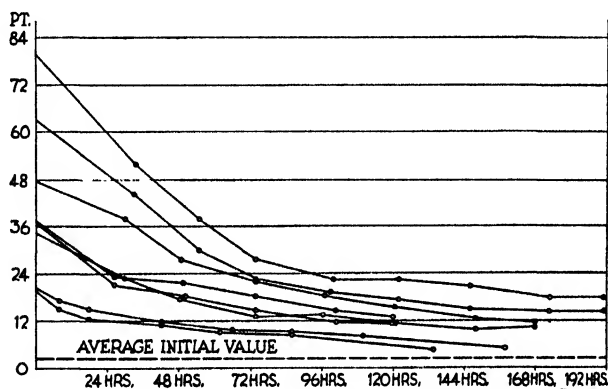


FIG. 4. The rate of disappearance of phosphatase from the serum of dogs given transfusions of jaundiced blood or serum.

#### DISCUSSION

Bile may contain anywhere from a few to several hundred units of phosphatase per 100 cc., depending upon the method of estimation and the source of bile. Armstrong, King, and Harris (8) reported high and widely varying values for the phosphatase content of bile with phenyl phosphate as the substrate for enzyme determination. They believe that serum phosphatase originates in bone and is excreted in the bile. Greene, Shattuck, and Kaplowitz (3) also recognized the existence of phosphatase in bile but they believed that this enzyme originated in the liver. We have found widely varying amounts of phosphatase in bile depending upon whether it was gallbladder or fistula bile, and if it was fistula bile, upon whether or not there was a hepatitis. In hepatitis, when the bile is of low viscosity and lightly colored, the phosphatase content is relatively low (10 to 25 units per 100 cc.).

The bile flow in a dog is several hundred cc. per day (200 to 500 cc.) and the phosphatase content, in our experience,<sup>\*</sup> 25 to 100 Bodansky units per 100 cc. of fistula bile. These values although much lower than those found by Armstrong, King, and Harris (8) still make the phosphatase contained in the daily output of bile several hundred units, and it seems unlikely that so much phosphatase could be derived from the serum when an excess is removed from the blood so slowly; particularly is this rate of removal slow as compared to that of the other constituents of bile.

Some relation between carbohydrate metabolism and serum phosphatase has been recognized. Kay (1) reported somewhat elevated values for serum phosphatase in clinical cases of diabetes. Freeman and Farmer (9) reported that a high carbohydrate-low protein diet causes an increase in the serum phosphatase of dogs previously fed a high protein diet. Bodansky (4) found that the ingestion of glucose or dextrin caused a transient increase in serum phosphatase as well as a decline in inorganic phosphorus. Binet and Pantrat (10), Shelling (11), and Freeman and Ivy (12) have all studied the effect of pancreatectomy upon the serum phosphatase of dogs. The latter investigators related the diabetic elevation of phosphatase to the prediabetic diet, and found that atrophy or subtotal pancreatectomy only slightly influenced the serum phosphatase. The present experiments do not permit one to decide as to the identity of the hyperglycemic substance contained in jaundiced blood. Certainly the animals receiving jaundiced blood showed a much more frequent, pronounced, and prolonged hyperglycemia than those receiving normal blood. The failure of some recipients receiving jaundiced blood to show a hyperglycemia is probably due to some difference between the recipients themselves; since it was noticed that of two recipients receiving blood from the same donor one might show a hyperglycemia, while the other would not. The pharmacologic effect of those constituents of bile that have been studied appears to be hypoglycemic (13, 14) upon injection into the circulation. Experiments with a purified phosphatase will be neces-

\* We are indebted to Dr. E. J. Kocour for allowing us to use bile from his bile fistula dogs.

sary before one can determine whether or not the hyperglycemia is due to the serum phosphatase elevation.

The serum inorganic phosphorus elevation was more constant and pronounced in the dogs receiving jaundiced blood. Experiments reported by Freeman and Farmer (9) indicated that an inverse relationship may exist between the serum phosphatase and the organic acid-soluble phosphorus of whole blood. If the serum phosphatase can reduce the concentration of organic acid-soluble phosphorus of the red blood cell, then the hydrolysis of this substrate may be responsible for the increased serum inorganic phosphorus. This increase is probably partially obscured in ten of the dogs by the simultaneous hyperglycemia, which normally has a tendency to depress the serum inorganic phosphorus.

#### SUMMARY

1. The serum phosphatase in normal dogs can be raised for several days by transfusing blood from a dog with obstructive jaundice. This slow disappearance of the injected phosphatase indicates that most of the phosphatase in bile does not come from the blood plasma, or that the phosphatase in jaundiced blood is bound to some substance which retards its excretion by the liver.

2. Changes of serum sugar and inorganic phosphorus occur in dogs following normal blood transfusion.

3. Marked hyperglycemia occurred in ten of fifteen dogs after transfusion with jaundiced blood.

4. Changes of serum inorganic phosphorus reciprocal to those of serum sugar occur in dogs given transfusions of jaundiced blood.

5. The relation between serum phosphatase and certain non-osseous factors which influence it is discussed.

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## BLOOD PLASMA PROTEINS IN RATS FOLLOWING PARTIAL HEPATECTOMY AND LAPAROTOMY\*

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The present communication is a study of the changes in albumin, globulin, fibrin, total protein, and albumin to globulin ratios in the plasma of rats following partial hepatectomy. Similar observations were made after laparotomy and in normal animals for comparison. Partial hepatectomy seemed particularly appropriate to the study of the rôle of the liver in the fabrication of plasma proteins for several reasons. In the first place, 70 per cent of the liver substance can be removed safely (1, 2). In the second place, regenerative changes following this operation have been studied in detail. For example, it has been shown that during the first 24 hours there is no mitotic activity and the water content of the liver stump is markedly decreased, probably from the increased deposition of fat. Evidences of regeneration are most marked on the 3rd day. The water content, number of cells, size of the cells, the chemical composition, and weight change rapidly then and the liver appears to be normal about the 14th day. In the third place, previous methods which made use of total hepatectomy (3), hepatotoxic chemicals (4), Eck fistula animals (5), and patients with liver disease (6) did not lend themselves to quantitative comparisons because of the lack of any uniformity in the pathological physiology.

Inbred male albino rats, 60 days of age, of Wistar stock, raised in this laboratory were used. Partial hepatectomy, under ether anesthesia, was carried out according to the procedure of Higgins and Anderson (1); 60 to 75 per cent of the total liver tissue was

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removed. Other animals were subjected to laparotomy alone, at which time the liver was handled and the intestines exposed for a period similar to that occupied by partial hepatectomy. The animals were fed a stock diet and were allowed food and water up to the time they were sacrificed. Intact animals served as controls. Blood was drawn from the abdominal aorta and was prevented from clotting by use of uniform concentrations of potassium oxalate. Globulin was precipitated with 22.2 per cent sodium sulfate and filtered through a Whatman No. 50 filter paper after incubation overnight. Fibrin was prepared for analysis from 1.0 cc. of plasma according to Cullen and Van Slyke's method (7). Protein nitrogen was determined by the micro-Kjeldahl procedure. Non-protein nitrogen was determined according to the procedure of Daly (8). Albumin and globulin were determined in each individual animal. Animals with gross signs of wound infection were not studied.

### *Results*

The results obtained on each rat subjected to laparotomy and partial hepatectomy are presented graphically in Figs. 1 to 5. The maximum and minimum variations in the "control" values for total nitrogen, albumin, globulin, and fibrin in the plasma of the intact rats are represented by two lines in each figure.

The values for fibrin in the laparotomized and partially hepatectomized rats are represented in Fig. 1. Since the concentration of fibrin is comparatively small, the scale has been magnified in Fig. 1. It can be seen that laparotomy exerts a profound influence, as is evidenced by the fibrin concentrations on the 1st day. These values decrease slowly and only two fall within the control range during the first 9 days, while the majority remain elevated until the 18th day. After hepatectomy there is a marked variation in the fibrin concentrations on the 1st day, but it is striking that removal of liver tissue has not been followed by the marked rise seen after laparotomy. The highest concentrations after partial hepatectomy were obtained during the 4th to 10th day. Thereafter there was a gradual general decrease in fibrin concentration, but the control range was not consistently reached by the 30th day after hepatectomy. The results seem to confirm previous evidence that the liver is of primary importance in the manufacture of fibrinogen.

The average of the albumin concentration is definitely decreased during the first 12 days after laparotomy (Fig. 2). By the 18th day all were within the control range. In the partially hepatectomized rats, the albumin concentrations decreased on the 1st day. On the 2nd and 3rd days, not a single value was within the control range. The albumin concentrations tended to remain low until

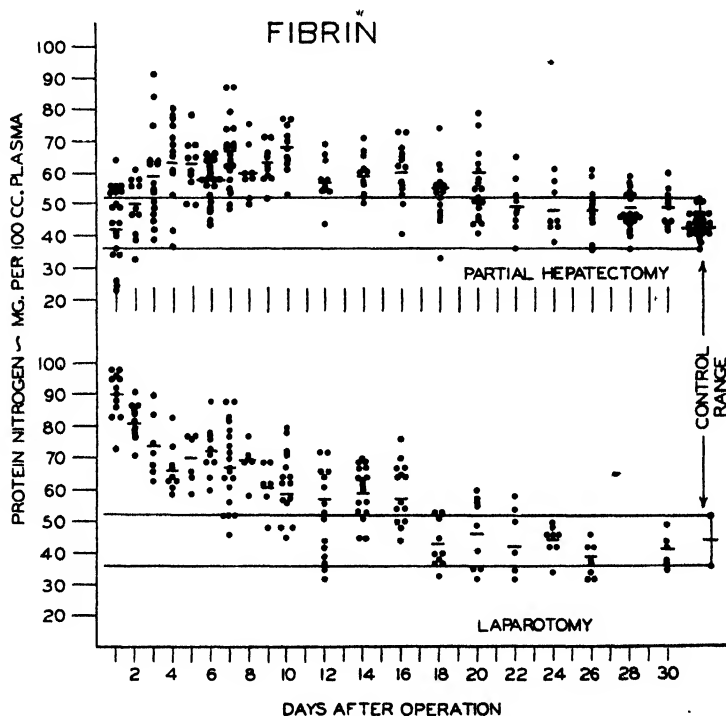


FIG. 1. Individual and mean values for fibrin nitrogen following partial hepatectomy and laparotomy.

the 18th day, when most of the values fell within the control range.

There appeared to be only a slight effect on the plasma globulin concentrations in the laparotomized animals during the first 6 days after operation (Fig. 3). After this time the globulin values tended to remain elevated until the end of the experiment on the 30th day. After partial hepatectomy the globulin is lowered on

the 1st day and is followed by an immediate rise to the control level on the 3rd day. After this there is an increase in the mean values, although the majority are within the control limits. The total nitrogen concentrations remained within the control range

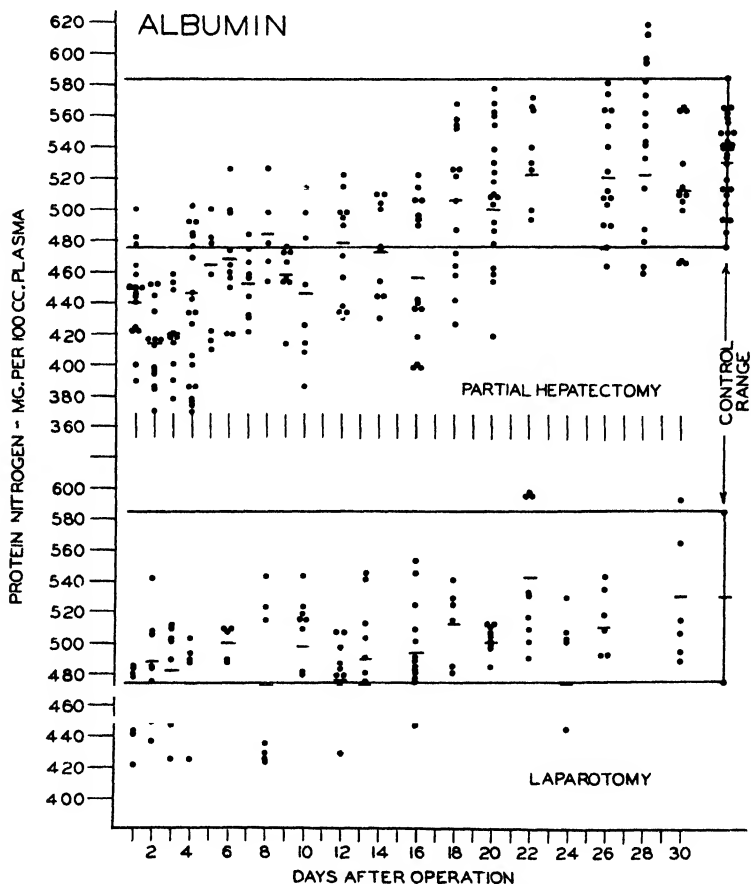


FIG. 2. Individual and mean values for albumin nitrogen following partial hepatectomy and laparotomy.

in the laparotomized rat with a few exceptions (Fig. 4). The effect of partial hepatectomy was manifested by results well below the control level during the first 5 days. Values fluctuated until the 16th day, after which practically all values were normal.

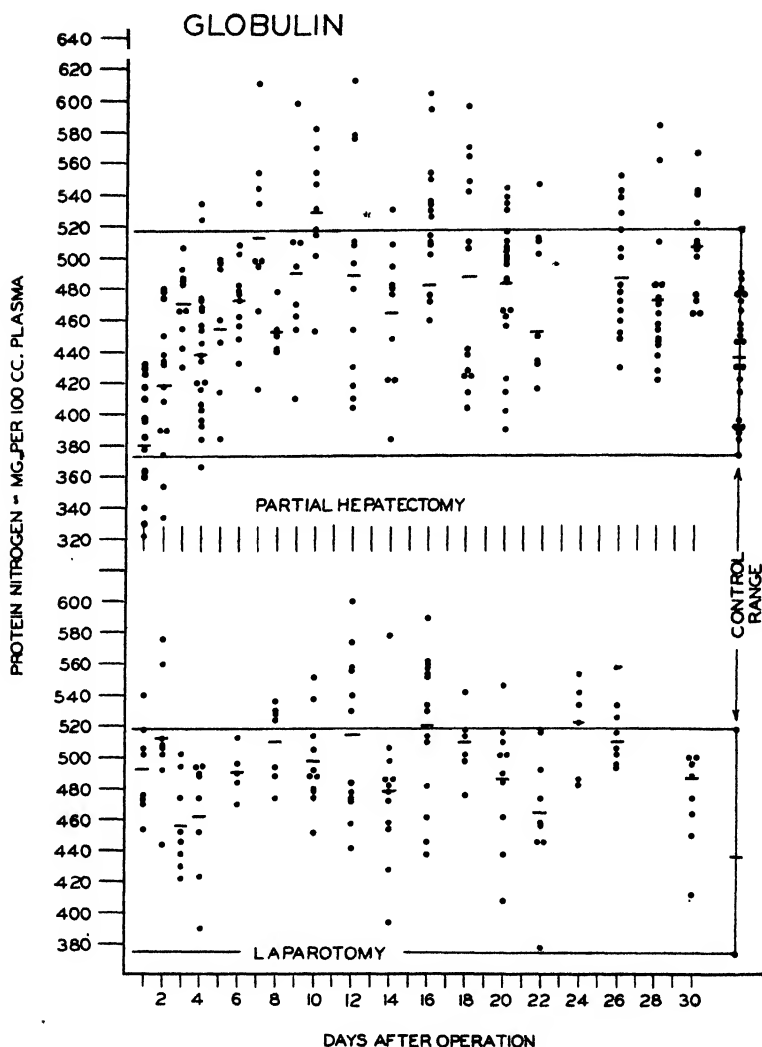


FIG. 3. Individual and mean values for globulin nitrogen following partial hepatectomy and laparotomy.

The albumin to globulin ratios of the laparotomized rats tended to remain low because of the proportionately greater increase in the globulin fraction. The ratios of the partially hepatectomized

rats remained normal during the 1st day, which demonstrates an equal effect on albumin and globulin. After this time the more

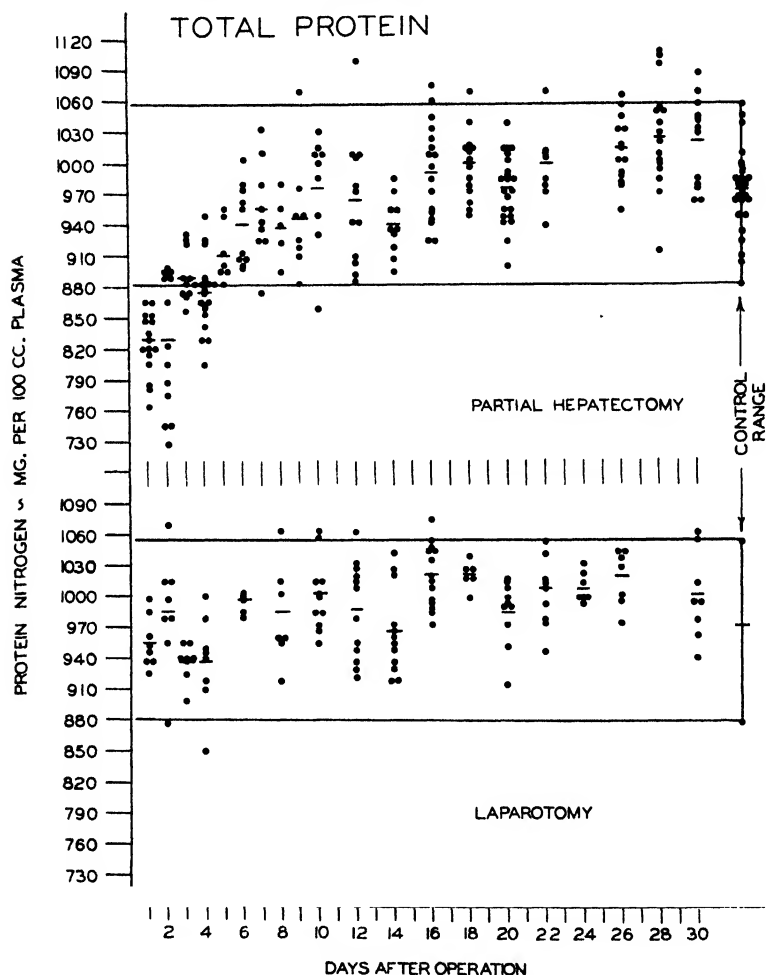


FIG. 4. Individual and mean values for total protein nitrogen following partial hepatectomy and laparotomy.

rapid regeneration of globulin in the respective animals manifested itself in the large number of low ratios. Most of the values were within the control range after the 18th day (Fig. 5).

*Comment*

The effects of laparotomy and partial hepatectomy on the albumin, globulin, and fibrin of the plasma are compared in Fig. 6, in which average values are plotted. The distribution around

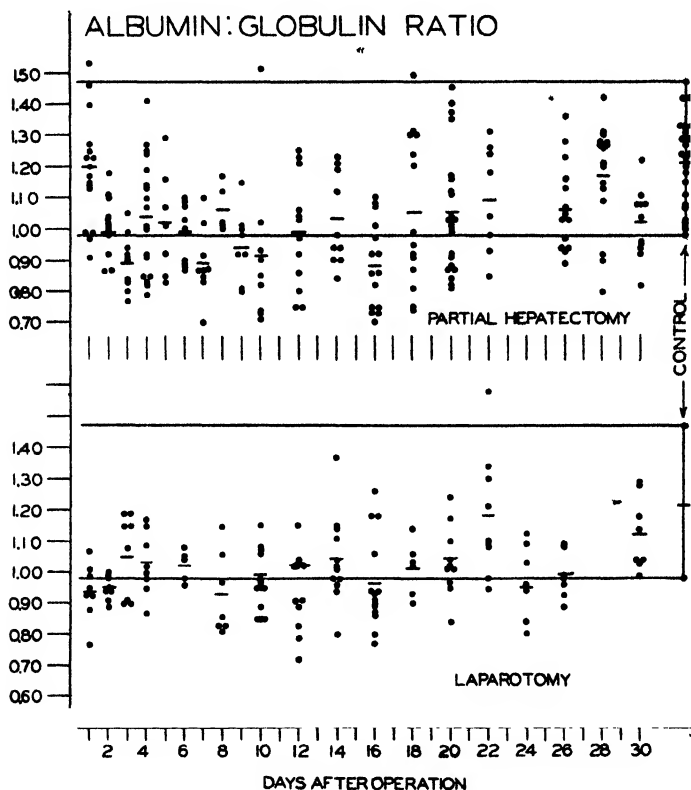


FIG. 5. Individual and mean values for the albumin to globulin ratio following partial hepatectomy and laparotomy.

the average of the "normal" values of the control intact rats was not essentially changed between the 60th and 90th days of life.

It will be seen that there is an obvious effect on the plasma proteins following the control laparotomy operation. There is an immediate and prolonged decrease of the plasma albumin which is difficult to explain unless one assumes that tissue damage reduces



the stimulus to albumin formation. There is an immediate and very sustained rise in the plasma globulin which indicates that the operative procedure of laparotomy alone stimulates globulin production at once and for a long period. There is an immediate but less sustained elevation of the plasma fibrin which appears attributable also to the tissue injury *per se*.

At first glance, it seems that the more marked alterations in the plasma proteins which occur after the partial hepatectomy might be ascribed to the greater operative procedure and the greater

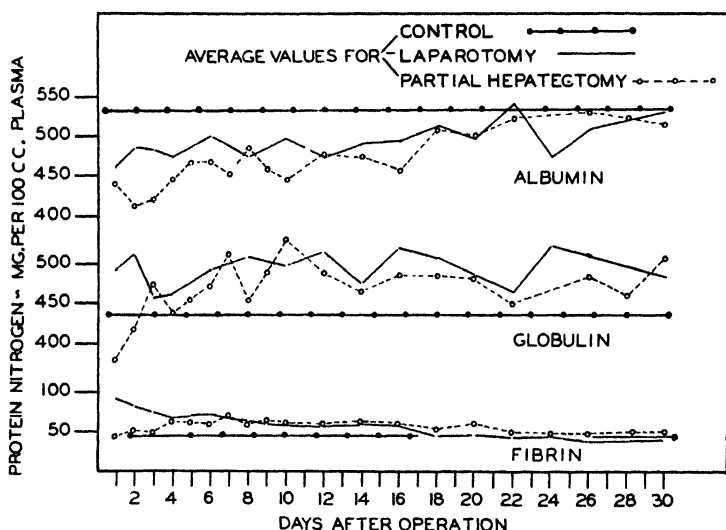


FIG. 6. Comparison of the mean values for albumin, globulin, and fibrin nitrogen in the partially hepatectomized and laparotomized rats.

postoperative disturbance. The effect on the plasma albumin is of the same kind as that following laparotomy alone and only the degree is accentuated. This further decrease in the plasma albumin cannot with certainty be attributed to the reduced liver substance rather than to the larger operation, but it would be difficult to devise any operative procedure which would serve as an adequate control to determine this point. On the other hand, the plasma globulin and fibrin do not increase over the laparotomy levels in the first few days after operation when the tissue injury

is at its greatest and the liver function at its least. The most probable explanation for this fact appears to be that the deficient liver cannot meet the stimulus for increased production of globulin and fibrin. If this is true, one must conclude that the liver is intimately associated with the fabrication of these substances.

The abnormal distribution of plasma albumin and globulin associated with pathological livers is assumed by most investigators to be the direct effect of hepatic insufficiency. This may possibly account for the decreased albumin but not for the increased globulin. In conditions in which the liver is not directly involved, such as malnutrition, renal diseases, and plasmapheresis, similar plasma protein changes are noted. At present there is no evidence for associating the liver directly with the plasma albumin- and globulin-regenerating mechanism.

#### SUMMARY

The concentration of blood plasma fibrin, albumin, and globulin was determined at frequent intervals in rats subjected to partial hepatectomy and to laparotomy alone.

A decrease in all these fractions was noted on the 1st day after partial hepatectomy. Fibrin and globulin concentrations increased on the 2nd and 3rd days and tended to remain above the normal values for the 30 day period of observation. The albumin concentration remained depressed until the 4th week after operation.

Laparotomy was followed immediately by increases in the concentration of fibrin and globulin and a decrease in the albumin. The globulin fraction remained high during the entire period of observation, and albumin and fibrin approached normal about the 18th day.

The most important effect of tissue injury and reduction of liver tissue is in the prolonged reduction of the plasma albumin which is accompanied by increased globulin concentrations.

These experiments do not necessarily support the thesis that the liver is directly involved in the fabrication of plasma proteins.

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## STUDIES ON KETOSIS

### XIII. DIURNAL CHANGES IN LIVER GLYCOGEN\*

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(Received for publication, November 22, 1937)

There is a considerable amount of evidence that cyclic variations may occur in the water, glycogen, and protein contents of the liver. However, there is a disagreement as to whether such alterations are to be ascribed to nutritional differences or whether they may also occur in fasted animals independent of the food intake.

From histochemical evidence on rabbits Forsgren (1) concluded that the liver exhibited an assimilatory cycle during the night, when glycogen storage was at its maximum and bile production was at the minimum. During the daytime a secretory cycle obtained, during which period the conditions were reversed. Such variations took place in spite of an abundant food supply at all times. Forsgren's results were confirmed by Agren, Wilander, and Jorpes (2) on rabbits, rats, and mice. Diurnal variations in susceptibility of mice to insulin were noted, about twice the dose of insulin being required to produce convulsions at night when the glycogen reserves were high as during the day. Moreover, about 20 per cent higher excretion of urinary nitrogen was noted during the night in fasted as well as non-fasted rabbits than during the day.

That such diurnal changes are independent of nutritional differences is challenged by Higgins, Berkson, and Flock (3). Although cyclic variations were noted with rats after a meal at 9 to 11 a.m., after which the water, glycogen, and protein content reached max-

\* This work was assisted by a research grant from the Rockefeller Foundation.

ima at 7 p.m. and again at 11 p.m., such a condition was not repeated on the next day if fasting continued. Moreover, these investigators later reported (4) that the liver glycogen was practically constant in various groups of rats 6 hours after feeding periods spaced at 2 hour intervals during the day and night. They conclude that there is little variation in the total weight of the liver or of its glycogen or water content during a 24 hour period except changes to be ascribed to food consumption. They also believe that the sources of glycogen other than from the gastrointestinal tract are probably insignificant in the cyclic changes.

Although there is a fairly close uniformity in the changes in weight of the water and glycogen when compared with the control levels, actually there are variations in liver glycogen of more than 50 per cent from the minimum mean to the maximum one in the results of Higgins, Berkson, and Flock (from 4.71 per cent, 3 a.m., to 7.16 per cent, 1 p.m.). Such wide variations in glycogen store might actually represent true cyclic variations or they might occur because other physiological factors were active which were not compensated for because of the relatively small number of animals in each group. For example, sex plays a most important rôle in determining the amount of glycogen which may be present, as noted for fasting animals by Deuel, Gulick, Grunewald, and Cutler (5) and for unfasted rats by Deuel, Hallman, and Murray (6). Moreover, age is an important factor and is associated with variations in glycogen store of unfasted rats (7), particularly in female rats about 3 months of age, when it reaches a minimum level. With animals 4 months of age and older, it seems from our more recent experiments that the sexual variation in liver glycogen is much less exaggerated.

In the present tests we have attempted to determine whether a diurnal variation could be noted in rats killed at various periods without fasting; we desired to ascertain also whether a constant level of liver glycogen could be demonstrated 12 hours after the administration of a standardized glucose test meal in animals previously fasted for 2 days. By killing our rats this length of time after the glucose was fed, the inequalities due to irregularities in rates of absorption are largely avoided. Lastly, we have sought to avoid complications caused by sex and age by making compar-

isons on rats of the same sex and approximate age. By using very large groups, the effects of other unknown physiological factors which may alter the glycogen deposition have been minimized.

### *Procedure*

In the first group of experiments (Table I) the rats were killed without fasting at 4 hour intervals from 8 a.m. during the day and night. In the second series of tests (reported in Table III), the rats were fasted a minimum of 48 hours (in some tests for 59 hours), at which time they were given 50 per cent glucose by stomach tube in a dose of 1 cc. per 100 sq. cm. of body surface. This dose could be well tolerated without any diarrhea. The body surface was calculated by the formula of Lee (8) from the weight after the preliminary fast period. The animals were killed under anytal anesthesia 12 hours later, the liver was removed, frozen in an ether-solid carbon dioxide mixture, and weighed. Glycogen was determined by the procedure of Good, Kramer, and Somogyi (9). In most of the tests thirty rats (fifteen males and fifteen females) were killed at 3 minute intervals during the period 45 minutes before and after the hour noted for the time of killing of each group.

Rats from our own colony previously on our stock diet were used. In the first group the rats were all approximately 4 months of age. They were so distributed that animals from a given litter were placed in the 'groups killed at various time intervals. The age of the animals used in the second series of tests was from 3 to 4 months. However, these were also so distributed that the ages averaged practically the same in the various time groups.

### *Results*

The average values of body weight, liver weight, and liver glycogen for the groups killed without fasting at various time intervals are summarized in Table I.

In order to obtain a measure as to the food intake, the whole gut was removed from the esophagus to the anus, and its weight was noted. The average values are recorded in Table II.

The data on the variations in liver glycogen found after the

TABLE I  
*Diurnal Variation in Liver Glycogen of Male and Female Rats Killed at Various Periods without Fasting*  
 Experiments carried out May 19 to 28, 1937.

Time killed	No. of experiments		Mean body weight		Mean liver weight		Liver weight $\times 100$ Body weight		Mean liver glycogen*	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
8.00 a.m.	20	19	gm. 264	gm. 189	gm. 9.77	gm. 6.82	3.70	3.61	per cent 4.54 $\pm$ 0.086	per cent 4.59 $\pm$ 0.149
12.00 m.	20	19	231	169	8.68	6.22	3.76	3.68	3.45 $\pm$ 0.192	3.29 $\pm$ 0.166
4.00 p.m.	18	20	264	188	8.33	5.95	3.15	3.16	1.88 $\pm$ 0.085	1.60 $\pm$ 0.085
8.00 "	20	20	262	188	8.56	5.94	3.27	3.16	2.64 $\pm$ 0.096	1.15 $\pm$ 0.060
12.00 "	17	20	256	172	8.32	5.67	3.25	3.30	3.43 $\pm$ 0.126	3.28 $\pm$ 0.180
4.00 a.m.	19	20	269	190	9.38	6.64	3.49	3.50	4.74 $\pm$ 0.135	4.37 $\pm$ 0.109
Total.....	114	118								
Average.....			257	183	8.85	6.21	3.45	3.39	3.47 $\pm$ 0.088	3.03 $\pm$ 0.106

\* Including probable error of the mean.

† The ratio of the mean difference to the probable error of mean difference of average glycogen of males (3.43 per cent) compared with that of females (3.03 per cent) is 3.17.

feeding of a standardized glucose meal are summarized in Table III. The first series of tests was carried out in June, 1937, on approximately thirty animals in each group (fifteen males and fifteen females), while an additional series throughout the 24 hours of a like number was carried out in September, and a third series throughout the night groups during the latter period. No essential differences were noted in the groups which might be ascribed to seasonal changes. Differences in the means of the various groups were minimized by increasing the number of observations.

TABLE II

*Average Weight of Gastrointestinal Tracts with Contents from Rats Killed without Fasting*

Time killed	No. of experiments		Average weight of gut and contents			
	Male	Female	Gm. per rat		Gm. per 100 sq. cm.	
			Male	Female	Male	Female
8.00 a.m.	20	19	19.38	14.95	7.34	7.92
12.00 m.	19	19	16.00	13.14	6.92	7.78
4.00 p.m.	18	20	18.46	14.22	6.99	7.57
8.00 "	20	20	20.44	15.78	7.82	8.40
12.00 "	17	20	17.10	14.81	6.68	8.62
4.00 a.m.	19	20	20.80	14.98	7.73	7.88

## DISCUSSION

There is a definite diurnal variation in the liver glycogen of unfasted rats which have an abundant food supply available at all times. With the male rats there is a maximum level at 4 a.m. (4.74 per cent), and a minimum value 12 hours later (1.88 per cent). A similar cycle was noted with female rats, although the peaks were delayed 4 hours (4.59 per cent at 8 a.m. and 1.15 per cent at 8 p.m.). It seems probable that these variations in glycogen are directly referable to the periodicity of food consumption. The rat is known to be a nocturnal feeder and according to our observations eats little during the daytime unless the food supply has become exhausted during the previous night.

That a variation in food intake occurred during the experiment was indicated by the fact that the stomachs of those killed during



TABLE III

*Liver Glycogen of Male and Female Rats Killed at Various Times during Day and Night 12 Hours after Administration of Glucose Following 48 Hour Fast*

Time killed	No. of experiments		Mean body weight		Mean liver weight		Liver weight $\times 100$ Body weight		Mean liver glycogen*		$\frac{M.D. \dagger}{P.M.M.D.}$
	Male	Fe- male	Male	Fe- male	Male	Female	Male	Female	Male	Female	
			gm.	gm.	gm.	gm.			per cent	per cent	
8.00 a.m.	29	29	199	146	6.25	4.92	3.13	3.37	3.37 $\pm$ 0.075	3.23 $\pm$ 0.085	1.27
12.00 m.	28	29	201	149	6.44	5.03	3.21	3.38	3.51 $\pm$ 0.049	3.11 $\pm$ 0.062	5.07
4.00 p.m.	25	29	187	148	6.43	5.14	3.43	3.47	3.53 $\pm$ 0.104	2.37 $\pm$ 0.062	9.59
8.00 "	36	36	201	146	6.37	4.67	3.17	3.20	3.15 $\pm$ 0.061	2.92 $\pm$ 0.066	2.59
12.00 "	43	45	200	145	6.12	4.77	3.06	3.29	3.61 $\pm$ 0.045	3.01 $\pm$ 0.069	7.32
4.00 a.m.	41	43	190	143	6.22	4.87	3.27	3.40	3.40 $\pm$ 0.067	3.08 $\pm$ 0.078	3.11
Total.....	202	211									
Grand average.....			196	146	6.28	4.88	3.21	3.34	3.43 $\pm$ 0.028	2.96 $\pm$ 0.032	10.93 ¶

\* Including probable error of the mean.

† Ratio of the mean difference to the probable error of mean difference of males compared with females. When this ratio exceeds 3, the results are considered statistically valid.

‡ One abnormally high value was dropped from the average.

§ One abnormally high value (6.42 per cent) and one abnormally low one (0.46 per cent) were dropped from the average.

|| Probable error of the mean computed for the grand average.

¶ Computed on values of the probable error of the mean for the grand average.

the night were filled, while those of the animals killed during the day were practically empty. The maximum weight of the gastrointestinal tracts of the male rats occurred at 8 p.m., while two minima were noted (12 p.m. and 12 m.). When considered in relation to the values of the remaining four groups, it would seem probable that the true minimum occurs at noon. On the other hand, the maximum and minimum weights of the gastrointestinal tracts of the females occurred in each case 4 hours later (i.e. at 12 p.m. and 4 p.m.). This agrees well with the fact that the maximum and minimum averages of liver glycogen in the females occurred in the periods 4 hours later than in the case of the males.

The actual change in weight of the gastrointestinal tract (based on gm. per 100 sq. cm. of body surface) is not very great. This is probably because the large intestine contained relatively less at the later night periods after the partial fast during the previous day, while the stomach and small intestine were largely filled from the food consumption during the night; on the other hand, there is a larger quantity of material still in the large intestine during the daytime as a result of the food consumption during the previous night in spite of the fact that the stomach and small gut have become largely emptied. The variations between these alternate periods of alimentation and fasting would be largely compensated for by the irregularities in the quantities of material present in the large gut. It would seem much more satisfactory to determine the extent of alimentation by a comparison of the weight of the stomach and small intestine rather than of the whole gastrointestinal tract as we carried it out. Unfortunately we realized the desirability of the latter procedure too late to apply it in these tests.

That cyclic variations in food intake are responsible for this variation in liver glycogen seems certain from the results on the second series of tests summarized in Table III. When similar doses of glucose based on surface area were fed to rats previously fasted for a sufficient time to reduce the liver glycogen to a base level, the glycogen deposited and retained 12 hours thereafter was practically identical at the various 4 hour periods studied during the day and night. In the tests on male rats the maximum variation from the grand average of 3.43 per cent of liver glycogen

(3.15 per cent at 8 p.m.) was only 8.2 per cent. With the females, five of the six groups approximate the grand average of 2.96 per cent, the maximum deviation being only 9.1 per cent higher. However, in the sixth group (4 p.m.), the average is only 2.37 per cent, which is 19.8 per cent less than the grand average and which is statistically different from any of the other groups. This value is the mean of two series of tests, that for those carried out on June 3, 1937, being 2.34 per cent (fourteen rats), while that for the second series on October 7 is 2.39 per cent (fifteen rats).

That sex is a factor which must be considered in such tests is emphasized again. In the experiments reported in Table I, in five of the six groups the glycogen is higher in the male rats than in the females, while the grand average of the glycogen in the males is significantly higher than in the females. In the second series of the present tests in which the prevailing age is somewhat under 4 months, the sex difference in liver glycogen which occurred in all groups was exaggerated. It was significantly more in four of the six groups. The ratio of mean difference to probable error of mean difference (10.93) for the grand average shows that the level of 3.43 per cent for glycogen in the males is highly significant when compared with that of 2.96 per cent for the females.

#### SUMMARY

A diurnal variation in the level of liver glycogen was observed in unfasted rats having access at all times to ample supplies of our stock diet. The liver glycogen varied from a maximum level in the males of 4.74 per cent at 4 a.m. to a minimum value of 1.88 at 4 p.m. The highest percentage of glycogen was found at 8 a.m. in the livers of the female rats (4.59 per cent), while the minimum level (1.15 per cent) occurred at 8 p.m. There is some evidence to indicate that these variations correspond to diurnal differences in food intake.

The level of liver glycogen 12 hours after a glucose test meal to male rats previously fasted for 2 days was remarkably constant at the 4 hour intervals studied during the 24 hour period. A similar uniformity was noted in five of the six periods investigated with female rats. In the group killed at 4 p.m. the variation seemed

greater than that which might be ascribed to experimental error. Possibly some physiological explanation such as increased activity may explain such differences rather than the assumption of a diurnal cycle. Since such is not the case with the males, it would seem highly improbable that it would apply to the females. Because of the marked regularity in the liver glycogen of male rats after the glucose test meal, it is concluded that the diurnal variation in this constituent is entirely of dietary origin.

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## THE METABOLISM OF PYRUVIC ACID IN VITAMIN B<sub>1</sub> DEFICIENCY AND IN INANITION\*

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The work of Peters (1) and of Sherman and Elvehjem (2) has made it appear quite certain that the primary biochemical lesion in vitamin B<sub>1</sub> deficiency is the failure of the organism to metabolize pyruvic acid. Lohmann and Schuster's recent announcement (3) that cocarboxylase is vitamin B<sub>1</sub> in combination with 2 molecules of phosphoric acid seems to localize the site of action of the vitamin with even greater certainty. The latter finding might lead to the conclusion that the rôle of the vitamin in animal tissues has been completely defined, and that *in vivo* the vitamin is phosphorylated and the resulting cocarboxylase makes possible the decarboxylation of pyruvic acid to acetaldehyde as in the case of yeast. Although the vitamin is probably intimately concerned with the decarboxylation, it appears from the work of Krebs and Johnson (4) that pyruvate is removed in a variety of ways and that the main reaction is really a dismutation in which 1 molecule of pyruvate is oxidatively decarboxylated, while the other molecule is reduced. The products from 2 molecules of pyruvic acid are thus CO<sub>2</sub>, acetic acid, and lactic acid. Krebs and Johnson (4) explain oxygen uptake in the presence of pyruvate as being due in part to the oxidation of the lactic acid formed in the dismutation. Krebs also stated that preliminary experiments indicated that vitamin B<sub>1</sub> was a coenzyme for the dismutation (5). It is not impossible that acetaldehyde is still an intermediate in the dismutation in which acetic acid is formed, but

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the work of Krebs does not appear to support this idea. In the case of the propionic acid bacteria, Wood, Stone, and Werkman (6) have suggested pyruvic acid hydrate as an intermediate. This would preclude acetaldehyde from the mechanism. In a consideration of the factors influencing pyruvate removal the important work of Weil-Malherbe (7) cannot be overlooked. He reported that pyruvic acid was removed more effectively in the presence of glucose than in the absence of accessory substrates.

The present work was begun with the purpose of clarifying some of the points in connection with pyruvate removal in animal tissues. In this paper, data will be presented which indicate that inanition of the animal is a complicating factor in the faulty pyruvate metabolism of polynuritic chick tissues *in vitro*. Evidence is presented which indicates that pyruvate removal is not a simple decarboxylation but that it is probably dependent upon the presence of other substrates.

#### EXPERIMENTAL

In these experiments polynuritis in chicks was produced in the same manner as in the work of Sherman and Elvehjem (8). White Leghorn chicks 4 to 6 weeks of age were shifted from a normal diet to one in which the vitamin B<sub>1</sub>-containing components were autoclaved (Ration 242A, Elvehjem (9)). The later experiments were made with chicks which had been grown for several weeks on the autoclaved ration containing 3 per cent of bakers' yeast. The yeast was then omitted and the birds came down with acute polynuritis in 1 to 3 weeks. The latter technique appears to be more desirable, since the chicks become accustomed to the autoclaved diet before the vitamin B<sub>1</sub> intake is reduced. Normal chicks for our control experiments were raised on our Ration 351 (10).

In all cases the tissue was prepared by the method of Potter and Elvehjem (11) in which the tissue is homogenized in a special device. The buffer was equimolar Na and K phosphate, and experiments were carried out at various molarities and pH values. 2 cc. of the homogenized tissue suspension containing 150 mg. per cc. were placed in 50 cc. Erlenmeyer flasks. The substrate was added and the final volume made to 3 cc. with the proper

buffer. The flasks were placed in a water bath at  $37^{\circ} \pm 0.2^{\circ}$  and were shaken at 120 oscillations per minute. The time which elapsed between the death of the animal and introduction of the flasks into the thermostat was 15 to 25 minutes.

Pyruvic acid was estimated by a modification of the method of Clift and Cook (12). It was found that the use of homogenized tissue obviated the necessity of grinding the tissue in a mortar with the protein precipitant. The contents of the flask were therefore washed directly into a centrifuge tube, precipitated with 1 cc. of 20 per cent trichloroacetic acid, diluted to 15 cc. with distilled water, and centrifuged. 3 cc. aliquots were taken for estimation on the basis of their bisulfite-binding capacity.

Later experiments designed to measure both oxygen uptake and pyruvate removal were conducted in Barcroft respirometers of the type described by Stare and Elvehjem (13).

Preliminary experiments involved (a) tissue concentration, (b) time of incubation, and (c) original pyruvate concentration. Potter and Elvehjem (11) showed that in systems requiring a coenzyme the oxygen uptake per gm. of tissue was dependent upon the concentration of the tissue suspension employed. If the system for the oxidative removal of pyruvic acid required a coenzyme, we should expect a "dilution effect" and less pyruvate should be removed per gm. of tissue at low concentrations than at higher concentrations. We have found this to be the case.

This may be considered further evidence for the view that a coenzyme is concerned in the oxidation of pyruvic acid. The dilution effect is evident mainly in concentrations below 100 mg. per cc. Potter and Elvehjem found that at tissue concentrations above 180 to 200 mg. per cc. the rate of oxygen uptake was limited by the viscosity of the suspension. Experiments therefore have been carried out at a level of 100 mg. per cc. of tissue suspension.

The optimum time period for studying pyruvate removal was determined by permitting tissue to incubate at  $37^{\circ}$  with constant aeration for varying periods of time. It was found that even in the presence of excess pyruvate the removal was not linear. In normal chick tissues pyruvate was removed rapidly during the 1st hour and slowly during the 2nd. At the end of 2 hours, removal had virtually ceased. In the tissues from fasted or poly-



neuritic birds, pyruvate removal had practically ceased at the end of 1 hour. However, all experiments have been conducted over a 2 hour period in order to permit comparison of the results.

Optimum pyruvate concentration was determined by incubating normal and polyneuritic kidney for 2 hours with various levels of pyruvate. Fig. 1 shows that the higher concentrations of pyruvate were toxic for both liver and kidney.

The pyruvic acid used in these experiments was prepared by double distillation of commercial pyruvic acid at 3 to 5 mm. and 38–45°. A water-clear liquid resulted which was diluted immediately to approximately 100 mg. per cc. for use as our stock solution. This was kept in the refrigerator. Aliquots were taken from this stock solution and prepared for use in the experiments

TABLE I  
*Effect of Method of Preparation of Sodium Pyruvate upon Purity of Product Obtained*

Concentration of pyruvic acid solution when neutralized	Pyruvic acid		
	Calculated from dilution	Found by analysis	Removed by 1 gm. liver per 2 hrs.
<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
11.2	28.00	22.20	1.77
	28.00	22.20	1.77
1.6	28.00	27.80	9.12
	28.00	27.80	9.12

immediately before the animal was killed. Nevertheless, variation in the toxicity of the neutralized pyruvate was observed. Further investigation showed that the procedure involved in neutralization affected both the bisulfite-binding capacity and the levels at which the substrate was toxic. When our stock solution was neutralized with 6 per cent NaOH and then diluted to the desired volume, a toxic product resulted (Table I). When the solution was diluted first to approximately 10 mg. per cc. and then neutralized, a product was obtained which showed a greater bisulfite-binding capacity and less toxicity. Apparently toxic polymers are formed when a concentrated solution of pyruvic acid is neutralized. The extreme lability of pyruvate has also been noted by Peters (1). In all subsequent experiments the

stock solution was diluted nearly to the concentration desired before neutralization. The procedure has been to add alkali to a pH of about 6.8 as shown by brom-thymol blue. The solution

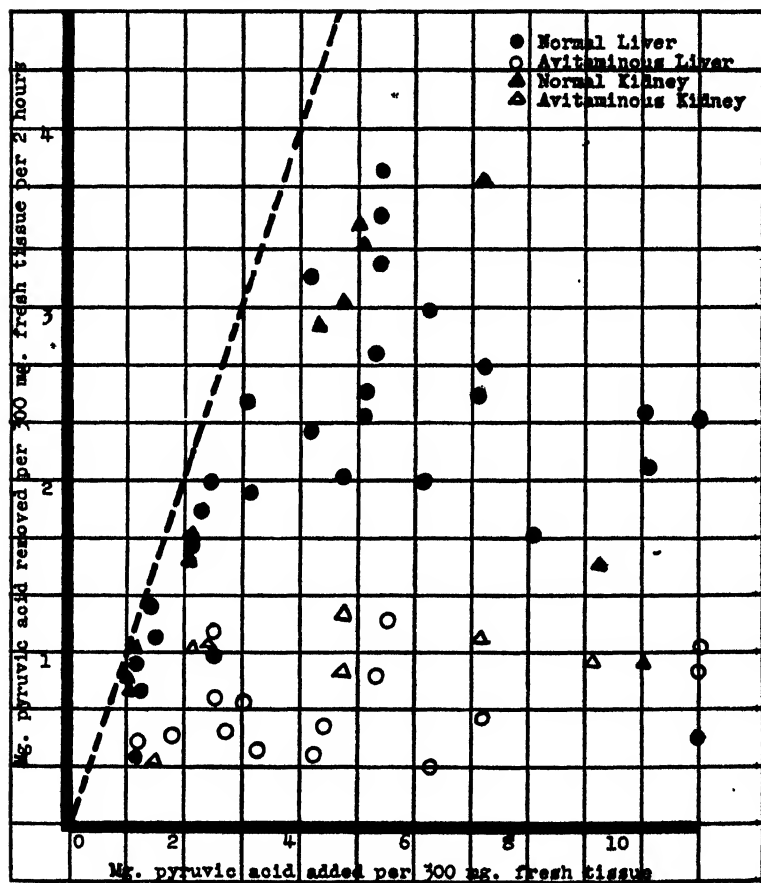


FIG. 1. Aerobic removal of various concentrations of sodium pyruvate by normal and avitaminous chick liver and kidney. The dotted line indicates 100 per cent removal.

was not used in the event that alkali was added beyond this point.

There remains the possibility that the results in Fig. 1 may have been complicated by the pH of the buffer. However, the

pyruvate was used in a relatively dilute solution and, furthermore, it was later shown that the pH of the resulting tissue suspension moved toward the acid range by from 0.3 to 0.6 pH unit when  $m/30$  buffer at pH 7.4 was used. It is possible that pyruvate could be obtained with a lower toxicity than is shown in Fig. 1, but we have used the optimum levels indicated, and in addition have used 0.1  $M$  buffer at pH 6.8 in the majority of our later experiments. Thus we are reasonably certain that pyruvate toxicity has not complicated any of our later work.

The inability of  $m/30$  phosphate buffer to maintain a desired pH caused us to study the effect of more concentrated buffers and various pH values upon pyruvate removal. All pH values were determined with a glass electrode. Using liver tissue and  $m/30$  buffer at pH 7.4 we found that the pH fell to about 6.8 very rapidly. Therefore the effect of molarity was studied by the use of liver tissue homogenized in  $H_2O$ , and in  $m/60$ ,  $m/30$ , and 0.1  $M$  Na and K phosphate at pH 6.8. Considering the removal at 0.1  $M$  strength as 100 per cent (17.5 mg. of pyruvate removed per gm. of fresh tissue per 2 hours) the results were as follows:  $H_2O$ , 25 per cent;  $m/60$ , 65 per cent; and  $m/30$ , 89 per cent. The 0.1  $M$  concentration thus appears to result in maximum removal, although it is little superior to  $m/30$  buffer. The experiment was repeated with solutions of NaCl and KCl and the results were very similar, indicating that the results were not due to specific ion effects. On the other hand, it is difficult to attribute the effects to changes in osmotic pressure, since in this technique the cells are almost completely disrupted.

The effect of pH upon pyruvate removal was studied in  $m/30$  buffer and figures are given for the pH value attained by the tissue suspension at the end of 2 hours incubation. In all cases the original pH of the buffer was shifted toward the optimum value which was within the range attained by unbuffered liver tissue. Considering the removal at pH 6.87 as 100 per cent (12.9 mg. removed per gm. of fresh tissue in 2 hours) the results were as follows: pH 6.5, 69 per cent; pH 7.04, 89 per cent; pH 7.12, 82 per cent; pH 7.22, 74 per cent.

A number of experiments have been carried out to establish levels for the removal of added pyruvate by normal and polyneuritic brain, liver, and kidney. These data are presented in

Tables II and III. It will be seen that in these experiments the liver and kidney of polyneuritic birds are much more seriously affected than is the brain. Polyneuritic liver and kidney removed 46 and 33 per cent as much pyruvate as normal liver and kidney, while polyneuritic brain removed 80 per cent as much as normal brain. It may also be noted that whereas normal liver and

TABLE II

*Aerobic Removal of Added Na Pyruvate by Normal Chick Tissues*

The results are expressed in mg. of pyruvate removed by 1 gm. of fresh tissue in 2 hours.

Liver		Kidney		Brain	
Pyruvate introduced	Pyruvate removed	Pyruvate introduced	Pyruvate removed	Pyruvate introduced	Pyruvate removed
m/30 Na + K phosphate buffer, pH 7.4					
24.80	17.15	13.50	9.35	24.80	5.00
17.20	11.10	12.96	6.40	17.30	5.57
17.70	11.15	15.60	9.90		
17.70	11.60	18.30	12.50		
0.1 M Na + K phosphate buffer, pH 6.8		16.80	8.45	0.1 M Na + K phosphate buffer, pH 6.8	
15.70	8.20	16.80	9.30	16.00	4.13
19.00	10.30	24.80	9.68	13.20	4.90
17.75	8.80	17.30	9.00	14.75	5.75
17.90	8.55	16.80	8.45	17.20	5.05
		0.1 M Na + K phosphate buffer, pH 6.8			
17.75	9.37	14.75	8.55		
14.75	7.90	17.20	6.60		
Average 18.01	10.41	17.19	8.67	17.21	5.07

kidney remove roughly twice as much pyruvate as brain, in polyneuritis the removal is very similar in all the tissues.

The large differences obtained with liver and kidney as contrasted with the small difference in the case of brain may be explained on the basis that carbohydrate intermediates are needed for pyruvate metabolism, as indicated below. Since neither polyneuritic nor normal brain possesses an appreciable amount

of carbohydrate reserve, they are both handicapped in an *in vitro* type of experiment and hence only a small difference can be shown between normal and affected tissues. It may also be mentioned that Brodie and Macleod (14) and Westenbrink (15) have shown that in acute polyneuritis the diminution in the vitamin B<sub>1</sub> content of the brain is less than in the other organs.

It has been noted by many observers that loss of appetite and severe inanition accompany vitamin B<sub>1</sub> deficiency. Chickens on

TABLE III

*Aerobic Removal of Added Na Pyruvate by Polyneuritic Chick Tissues*

The results are expressed in mg. of pyruvate removed by 1 gm. of fresh tissue in 2 hours.

Liver			Kidney			Brain		
Days with symptoms	Pyruvate introduced	Pyruvate removed	Days with symptoms	Pyruvate introduced	Pyruvate removed	Days with symptoms	Pyruvate introduced	Pyruvate removed
M/30 Na + K phosphate buffer, pH 7.4								
2	17.30	4.10	2	15.60	3.26	1	17.30	4.01
2	16.60	3.95	1	18.30	4.34			
			2	15.70	2.76			
0.1 M Na + K phosphate buffer, pH 6.8			1	16.30	5.05	0.1 M Na + K phosphate buffer, pH 6.8		
1	15.60	6.05	3	16.00	4.25	1	15.40	4.85*
2	15.20	5.30	3	11.00	1.36	4	14.40	3.50
3 hrs.	15.50	7.25	2	11.00	1.88			
4 days	14.40	1.65	1	17.30	3.50			
Average	15.77	4.72		15.61	3.08		15.70	4.09

\* Run for 100 minutes.

our polyneuritic ration lost 20 to 45 per cent of their body weight before the development of opisthotonic symptoms. Rydin (16) reported that in pigeons on a ration of polished rice losses of 43 per cent of the body weight were obtained before head retractions ensued. He also reported (17) that the decreased oxygen uptake of skeletal muscle, liver, and kidney noted in polyneuritis was due to inanition. Galvao and Pereira (18) have shown a low O<sub>2</sub> uptake of skeletal muscle from avitaminous birds. Respiration

was not restored to normal when the bird was cured by feeding of vitamin concentrates. However, when the animal was cured by the vitamin and then fed, the  $O_2$  uptake was restored to normal.

In view of the complicating effect of inanition, we felt that it would be desirable to study pyruvate removal in tissues from fasted chickens. The results obtained with various tissues are shown in Table IV. It may be seen that the ability of liver to remove added pyruvate is very seriously impaired in fasting. Kidney is somewhat less affected, and brain is unaffected. The

TABLE IV

*Effect of Inanition on Ability of Chick Tissues to Remove Added Na Pyruvate Aerobically*

The results are expressed in mg. of pyruvate removed by 1 gm. of fresh tissue in 2 hours. All experiments in 0.1 M phosphate buffer, pH 6.8.

Liver			Kidney			Brain		
Time fasted	Pyruvate introduced	Pyruvate removed	Time fasted	Pyruvate introduced	Pyruvate removed	Time fasted	Pyruvate introduced	Pyruvate removed
hrs.			hrs.			hrs.		
44	15.10	3.60	172	15.30	3.00	172	15.30	5.90
48	17.90	2.70	144	16.00	3.59	144	16.00	5.20
96	17.35	4.67	120	15.95	4.20	120	15.95	5.16
120	14.10	4.50	60	16.35	6.12	60	16.35	6.14
196	18.85	1.90	99	15.86	4.97	99	15.86	5.48
Average..	16.66	3.47		15.89	4.38		15.89	5.58

results obtained with liver and kidney parallel those obtained with polyneuritic liver and kidney almost exactly. The fasting appeared to have no effect upon the brain, while in polyneuritis the brain was 80 per cent of normal.

The above experiments indicated that other substrates were involved in the mechanism of pyruvate removal. It was decided to test this idea by restoration of the carbohydrate content of the liver by oral administration of glucose solution. The following experiment was devised. Chicks were fasted for various periods of time. Birds were then paired on the basis of weight loss. The first bird was killed and its liver was removed and

homogenized. Aliquot portions of the tissue suspension were then placed in Barcroft respirometers and the following measurements made: oxygen uptake with and without pyruvate, and pyruvate removal. The original tissue suspension was also analyzed for total and reducing carbohydrate by a modification of the Hagedorn-Jensen method (19).<sup>1</sup> The second bird was given 0.5 gm. of glucose per 100 gm. of body weight by pipette. 3 hours later, the bird was killed and its liver was removed and studied as above. The results are given in Table V. It was found that glucose administration resulted in a complete restoration of the ability of the liver to remove pyruvate. Furthermore this increased removal was paralleled by an increase in the carbohydrate content of the tissue and by an increase in the residual respiration as well as the respiration in the presence of pyruvate. These results indicate that the presence of carbohydrate in liver has a definite relationship to the ability of the liver to metabolize pyruvic acid.

Our attention was next turned to the effect of glucose administration upon the livers of polyneuritic birds. The technique employed was identical with that used for the fasted normal birds. The results are given in Table V. Polyneuritic birds which received glucose had an average of 3.82 per cent total carbohydrate and 0.75 per cent glucose. Since the increase upon hydrolysis is predominantly glycogen, these results appear to confirm those of Schrader (20) who reported that polyneuritic birds retain the ability to synthesize glycogen from ingested glucose. The increase in carbohydrate content which we have observed is paralleled by an increase in the ability of the tissue to remove pyruvate as well as an increase in the residual oxygen uptake and the oxygen uptake due to pyruvate. This increased pyruvate removal

<sup>1</sup> Immediately following homogenization (within 10 minutes after death) an aliquot of the homogenized tissue suspension was precipitated with  $\text{Zn}(\text{OH})_2$  and centrifuged. One portion of the filtrate was analyzed for glucose in the usual manner (19). Another portion was hydrolyzed for 2 hours at  $100^\circ$  with 0.1 volume of 25 per cent  $\text{HCl}$ . The hydrolysate was exactly neutralized and analyzed for glucose as usual. Results are expressed as total carbohydrate and as glucose. In samples which gave a large difference in the two titrations the protein-free filtrates showed a marked opalescence due to glycogen. Neither glycogen nor maltose affects the ferricyanide reagent.

TABLE V

*Effect of Glucose Ingestion upon O<sub>2</sub> Uptake, Pyruvate Removal, and Carbohydrate Storage by Livers from Polyneuritic and Fasted Chicks*

Experiments in 0.1 M Na + K phosphate buffer, pH 6.8. The pyruvate removal is expressed in mg. removed by 1 gm. of tissue in 2 hours.

Time fasted	Weight loss	O <sub>2</sub> uptake per gm. per 2 hrs.			Pyruvate removed	Total carbohydrate	Glucose
		No substrate	With pyruvate	Difference			

Fasted chicks							
hrs.	per cent	μl.	μl.	μl.	mg.	per cent	per cent
65	21.0	1630	3920	2290	11.60	0.95	0.75
90	24.5	1135	2080	945	8.25	0.74	0.63
72	24.0	640	1415	775	6.45	0.56	0.58
120	28.0	724	878	154	7.70	0.66	0.59

Fasted chicks and glucose therapy							
65	23.6	1405	5520	4115	13.20	3.85	1.00
90	22.4	2630	6800	4170	12.00	4.52	1.48
120		2290	6050	3760	12.85	3.87	0.99

Polyneuritic chicks							
Time with symptoms							
3	31	1260	3480	2220	5.35	0.73	0.73
10	37	1545	4860	3315	9.35	0.93	0.86
12	15	700	2500	1800	3.10		
24	32	1250	4205	2955	5.45	1.51	0.93
6	21	1810	4740	2930	6.57	1.62	1.07
12	23	860	2010	1150	2.75	0.56	0.55
24	16	833	2720	1887	4.12	0.85	0.47
3	20	1440	3900	2460	6.05	1.00	0.66

Polyneuritic chicks and glucose therapy							
36	36.8	1800	4505	2705	7.40	1.05	0.99
24	29.0	1790	5140	3350	8.25	3.78	1.38
12	18.5	1360	5455	4095	9.15		
8-12	21.0	1775	5900	4125	9.19	4.70	1.00
36-48	21.0	1885	4100	2215	6.80	4.60	0.75
12	12.5	1830	4765	2935	9.65	4.24	0.92
24-36	18.5	2315	5550	3235	8.90	4.57	1.12



did not represent 100 per cent restoration, since on the average it was about 80 per cent of that observed in normal liver.

#### DISCUSSION

The results of our experiments indicate that the metabolism of pyruvic acid in tissues from normal birds is closely associated with the presence of other substrates which are probably intermediates in glycogen or glucose metabolism.

Weil-Malherbe (7) has studied the anaerobic production of succinic from pyruvic acid in brain. He found that added glucose increased the formation of succinic acid far above summation in the case of slices. However, there was no glucose effect with minced brain. We have made one attempt to secure increased pyruvate removal in the liver from a fasted bird by the *in vitro* addition of glucose, with negative results. It seems possible that homogenized tissue may not be able to convert glucose into intermediates which will aid in the removal of pyruvate, while if the glucose is given *in vivo*, the resulting glycogen may form intermediates which will aid in the pyruvate removal.

It may be argued that the large differences which we have obtained between birds which were given glucose and birds which were polyneuritic or fasted are a function of the technique employed and that no analogy may be drawn between our work and *in vivo* conditions. We have, however, further evidence for the idea that pyruvate metabolism is impaired during fasting. Sherman and Elvehjem (21) showed that polyneuritic birds were unable to remove injected pyruvate from the blood stream as rapidly as normal birds. We have repeated these experiments using fasted control birds, and find that in the latter there is also a lowered rate of pyruvate removal. The *in vivo* differences are not as great as those obtained *in vitro*, however.

We are not as yet able to offer any further explanation of the results obtained in fasting. After this work was completed Rosenthal (22) found that the metabolism of pyruvate and lactate was impaired in fasting, while succinate was not affected. The cytochrome-indophenol oxidase system cannot therefore be affected by fasting. Rosenthal suggests that possibly other enzymes or their components are affected and so alter the metabolism of lactate and pyruvate. Our results indicate either that

the enzyme and coenzyme systems for the metabolism of pyruvic acid are not affected in fasting, or that restoration of these systems is rapidly affected by the feeding of glucose. We have observed that the kinetics of the oxygen uptake of homogenized tissue may be very different from that of intact cells. In the latter the oxygen uptake is virtually linear in the absence of added substrate for 60 to 90 minutes. This may well be due to a limiting rate of oxygen diffusion. In homogenized tissue, cell barriers are broken down and the residual respiration is never linear. During the first 20 minutes the rate may be 2 or 3 times as rapid as with minced tissue in which the cells are predominantly intact, but it soon falls to a rate which is considerably lower. This first rapid uptake may be associated with the oxidation of compounds whose existence in the reduced form is essential to normal pyruvate removal. The presence of varying amounts of certain substrates may appreciably alter the rate at which these essential components of the system are converted to the oxidized form. Studies are being made in this laboratory in order further to elucidate differences which may exist between homogenized and intact tissues with respect to pyruvate metabolism.

There has not appeared in the literature any evidence for the existence of a catatorulin effect with tissues other than brain and kidney (23) and heart (8). Yet Simola (24) has demonstrated a lowered cocarboxylase content of the livers of rats on a vitamin B complex-deficient diet.<sup>2</sup> Two interpretations of the absence of a catatorulin effect seem possible. Either vitamin B<sub>1</sub> or cocarboxylase is stored in the liver, but is not utilized there, or some, as yet unknown difficulties have made it impossible to secure a catatorulin effect in this tissue. Our experiments indicate that the ability of liver tissue to metabolize pyruvic acid is seriously impaired in polyneuritis, and that vitamin B<sub>1</sub> functions in liver metabolism. It would seem, therefore, that polyneuritis is more properly conceived as a disease in which the carbohydrate metabolism of the entire organism is deranged, rather than as a specific disease in which the nervous system alone is affected.

<sup>2</sup> We have repeated the experiments of Simola and find that the technique employed fails to measure cocarboxylase exclusively, since the atiozymase preparation is able to synthesize cocarboxylase *in vitro* from added vitamin B<sub>1</sub> in the presence of liver *Kochsafi*.

## SUMMARY

1. The oxygen uptake with and without added pyruvate, as well as the pyruvate removal in liver, kidney, and brain from normal, fasted, and polyneuritic chicks has been investigated by the homogenized tissue technique.

2. It has been found that a dilution effect is observed with chick tissues when pyruvate is used as substrate. This is offered as further evidence of the need for a coenzyme for the metabolism of pyruvic acid.

3. It has been found that under our conditions the ability of kidney, liver, and brain to utilize pyruvate as a substrate is impaired in polyneuritis. The derangement is greater in the case of brain.

4. The liver and kidney from fasted birds show a decreased ability to remove added pyruvate. The brain does not show this fasting effect. Pyruvate removal by the liver and kidney of fasted birds approximates that found in polyneuritis.

5. The oral administration of glucose to fasted birds resulted in restoration of the ability of liver to remove pyruvate. Similar feeding of glucose to polyneuritic birds resulted in the deposition of liver glycogen and an increased ability to remove pyruvate though normal values were not reached.

6. The failure to obtain complete restoration of the ability to remove pyruvate in the case of polyneuritic birds fed glucose shows that inanition is not the sole explanation of the deranged pyruvate metabolism, although it certainly is a complicating factor.

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## BUFFER INTENSITIES OF MILK AND MILK CONSTITUENTS

### III. BUFFER ACTION OF CALCIUM CITRATE\*

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The buffer actions of casein and of calcium phosphate have been discussed in previous papers of this series (7). Because the molar concentration of total citrate is only approximately one-half that of phosphate in fresh milk, it would seem that the buffer action of the citrates would be of minor importance. However, since the ionic equilibria involving protein, phosphate, and citrate are all interdependent by way of the calcium ion concentration, the contribution of citrate to the buffer action should not be dismissed without investigation of its mechanism. In this paper equations describing the buffer curve of calcium citrate are derived and experimental data on the buffer action are given.

The peculiar behavior of citrate ions in the presence of calcium ions has been investigated by many workers in recent years. Most of the evidence supports the hypothesis that calcium and citrate form a complex ion. This evidence has been summarized by Hastings and coworkers (3), who offer the hypothesis that the complex ion is  $\text{CaC}_6\text{H}_5\text{O}_7^-$ . They postulated practically complete dissociation of tricalcium citrate in dilute solution according to the equation



and partial dissociation of the complex ion



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\* Presented in part before the Division of Biological Chemistry of the American Chemical Society at Rochester, New York, September, 1937.

They substantiated this hypothesis by determining the dissociation constant for this secondary reaction and finding it satisfactorily constant.

If the concentration of calcium ions in fresh milk is of the order of  $3 \times 10^{-4}$  moles per liter (6), and the solubility product of tricalcium citrate, expressed as if calcium and trivalent citrate ions were the ions involved, is of the order of  $1 \times 10^{-8}$ , as determined roughly by the author, then the concentration of the trivalent citrate ion in milk cannot be greater than about 0.0005 of that necessary for precipitation of tricalcium citrate. Hence the buffer equations are derived on a homogeneous basis only. Even though for use in other situations it might seem desirable to have the equations involving heterogeneous equilibria, it is futile to derive them until the mechanism of precipitation and the solubility constants are known.

In order that details of changes in curvature might be rendered sufficiently apparent for discussion, it was necessary in the experimental derivation of the buffer curves to use solutions 10 times as concentrated with respect to total citrate as is normal milk. Consequently, in many of the separately prepared samples on which pH determinations were made, calcium citrate precipitated after a time, but there was no difficulty in obtaining pH values before precipitation began. There was usually precipitated immediately a gelatinous mass, which readily redissolved on shaking. This was evidently the  $\text{Ca}_3(\text{C}_6\text{H}_5\text{O}_7)_2 \cdot 16\text{H}_2\text{O}$  mentioned by Chatterjee (2). The time elapsing before appearance of the stable granular precipitate was at least half an hour in the most alkaline solutions, and considerably longer in those in which the degree of supersaturation was less. From pH values determined later, when solubility had been attained, it was possible to construct the branches of the titration and buffer curves describing the heterogeneous equilibria. These appear in the plots, but, since they are not involved in milk equilibria, are not discussed here.

Fig. 1 shows titration curves drawn from data of preliminary work involving only twenty pH determinations per series. Although only tri- and dicalcium citrates are considered in the later discussion, curves for monocalcium citrate and for calcium-free citric acid are included to show the modifying influence of an increased proportion of calcium. Figs. 2 and 3 show buffer curves

derived graphically from the slopes of more detailed titration curves on 0.05 M tri- and dicalcium citrate solutions. Approx-

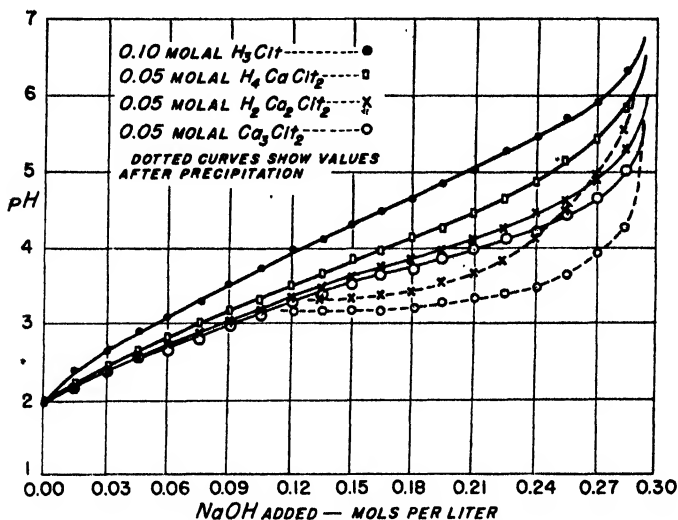


FIG. 1. Experimental titration curves for citric acid and three calcium citrates.

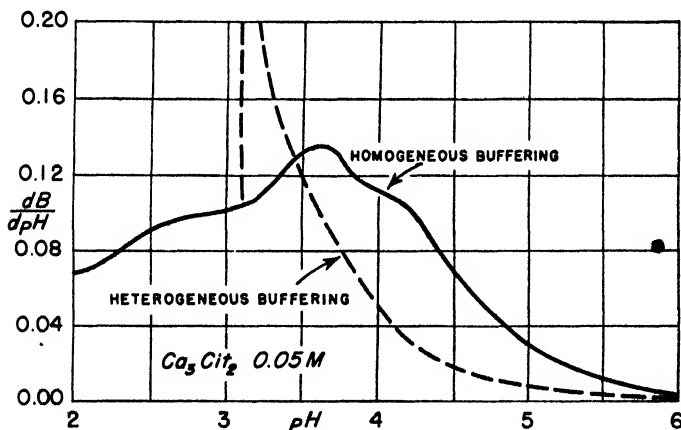


FIG. 2. Experimental curve for buffering by 0.05 M tricalcium citrate

mately 60 pH determinations were made on separately prepared samples for each titration series. Slopes were read from titra-



tion curves plotted on a large scale. These curves differ remarkably from the buffer curve for citric acid given by Hastings and Van Slyke (4), which shows its principal maximum in the region of pH 5.0. The presence of calcium obviously brings the maximum to regions of lower pH value.

Since calcium, when present in citrate solutions, is clearly involved in the buffer action, it seemed desirable to derive buffer equations on the basis of the Hastings-McLean hypothesis and to determine whether the experimentally derived curves are satisfactorily described by the theoretically derived equations.

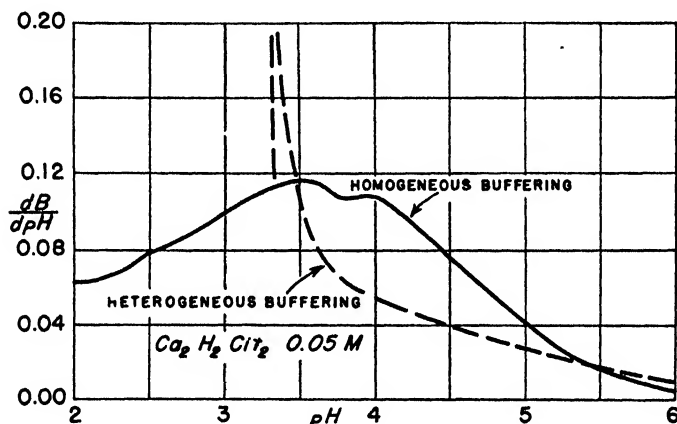


FIG 3. Experimental curve for buffering by 0.05 M dicalcium citrate

*Derivation of Equations for Hydrogen Ion Buffer Intensity of Calcium Citrate Solutions Having No Solid Phase Present*—For purposes of convenience and economy of space the following symbols are used.

- $C_4$  = concentration of  $\text{CaC}_6\text{H}_5\text{O}_7^-$  (in moles per liter)  
 $C_3$  = " "  $\text{C}_6\text{H}_5\text{O}_7^-$   
 $C_2$  = " "  $\text{HC}_6\text{H}_4\text{O}_7^-$   
 $C_1$  = " "  $\text{H}_2\text{C}_6\text{H}_5\text{O}_7^-$   
 $C$  = " "  $\text{H}_3\text{C}_6\text{H}_5\text{O}_7$   
 $S$  = sum of concentrations of all forms of citric acid in solution  
 $\text{Ca}$  = concentration of calcium ions  
 $R$  = sum of concentrations of all forms of calcium in solution  
 $H$  = concentration of hydrogen ions  
 $\text{pH}$  =  $\log 1/H$

OH = concentration of hydroxyl ions

$B$  = number of moles of completely dissociated monoacid base added per liter of total volume of solution

$k_1$ ,  $k_2$ , and  $k_3$  = respectively, the first, second, and third dissociation constants of citric acid

$k_4$  = dissociation constant of  $\text{CaC}_2\text{H}_3\text{O}_7^-$

$K = k_4/k_1k_2k_3$

$w$  = the function  $k_1k_2k_3 + k_1k_2\text{H} + k_1\text{H}^2 + \text{H}^3$

$v$  = " "  $dw/d\text{H}$ , or  $k_1k_3 + 2k_1\text{H} + 3\text{H}^2$

In the derivation of an equation for  $dB/d\text{pH}$ , the buffer index, the concentration of some form of citrate and the derivative of the concentration of this form with respect to hydrogen ion concentration appear as variables that are not directly determinable. In this derivation,  $C_4$  has been chosen as the concentration in terms of which the concentrations of other forms are expressed. In the following development, the values of  $C_4$  and  $dC_4/d\text{H}$  have been derived first and, subsequently, the value of the buffer index.

$C_4$  will be, in any solution of calcium citrate, a function of the concentrations of total calcium, total citrate, and hydrogen ions.

$$(3) \quad C_4 = f(R, S, \text{H})$$

In a given calcium citrate solution, with no solid phase present, the concentrations of total calcium and total citrate will be constant. Consequently,  $\text{H}$  will be the only independent variable in the above expression. The value of this function may be derived from the expressions for the successive dissociations of citric acid, the dissociation of the univalent calcium citrate ion, and the additive relationships of the various forms of calcium and citrate.

Calcium ion concentration is not readily determinable, except in a narrow range of concentrations and under special conditions. Hence it is eliminated between the equations for the dissociation constant of the calcium citrate complex and the sum of the forms of calcium.

$$(4) \quad \frac{\text{Ca}C_3}{C_4} = k_4 \qquad (5) \quad R = C_2 + C_4$$

From these equations

$$(6) \quad C_4 = \frac{C_4 k_4}{R - C_4}$$

The equations for the ionization constants of citric acid are

$$(7) \quad \frac{HC_1}{C} = k_1 \quad (8) \quad \frac{HC_2}{C_1} = k_2 \quad (9) \quad \frac{HC_3}{C_2} = k_3$$

By substitution of Equation 6 in No. 9, then Equation 10 in No. 8, and Equation 11 in No. 7

$$(10) \quad C_2 = \frac{HC_1 k_4}{(R - C_1) k_3}$$

$$(11) \quad C_1 = \frac{H^2 C_4 k_4}{(R - C_1) k_2 k_3} \quad (12) \quad C = \frac{H^2 C_4 k_4}{(R - C_1) k_1 k_2 k_3}$$

By convention

$$(13) \quad S = C_4 + C_3 + C_2 + C_1 + C$$

By substitution in Equation 13

$$(14) \quad S = C_4 + \frac{C_4 k_4}{R - C_4} + \frac{HC_4 k_4}{(R - C_4) k_3} + \frac{H^2 C_4 k_4}{(R - C_4) k_2 k_3} + \frac{H^3 C_4 k_4}{(R - C_4) k_1 k_2 k_3}$$

By rearrangement and introduction of  $w$  and  $K$

$$(15) \quad C_4^2 - (R + S + wK) C_4 = -RS$$

Whence

$$(16) \quad C_4 = \frac{R + S + wK - \sqrt{(R + S + wK)^2 - 4RS}}{2}$$

By differentiation

$$(17) \quad \frac{dC_4}{dH} = \frac{vK}{2} \left( 1 - \frac{R + S + wK}{\sqrt{(R + S + wK)^2 - 4RS}} \right)$$

In any solution containing only the ions derived from calcium citrate and water, the ionic equivalence may be expressed in terms of molar concentrations of the participating ions, as follows:

$$(18) \quad H + 2Ca = OH + C_1 + 2C_2 + 3C_3 + C_4$$

If  $B$  moles of completely dissociated monoacid base are added per liter, each of the ionic concentrations is changed, and the ionic equivalence may be represented by a new equation

$$(19) \quad H + 2Ca + B = OH + C_1 + 2C_2 + 3C_3 + C_4$$

By rearrangement and substitution of values

$$(20) \quad B = \text{OH} - \text{H} - 2R + 3C + \frac{C_4 k_4 (\text{H}^2 + 2k_2 \text{H} + 3k_2 k_3)}{k_2 k_3 (R - C_4)}$$

By differentiation with respect to H

$$(21) \quad \frac{dB}{dH} = \frac{d\text{OH}}{dH} - \frac{dH}{dH} + \frac{2C_4 k_4 (\text{H} + k_2)}{k_2 k_3 (R - C_4)} \\ - \frac{dC_4}{dH} \left( 3 + \frac{Rk_4 (\text{H}^2 + 2k_2 \text{H} + 3k_2 k_3)}{k_2 k_3 (R - C_4)^2} \right)$$

Between pH 3 and pH 9,  $d\text{OH}/dH - dH/dH$  is relatively so small that it may be omitted. By definition

$$(22) \quad \frac{dH}{dpH} = -2.303H$$

Therefore

$$(23) \quad \frac{dB}{dpH} = -\frac{4.606 C_4 k_4 H (\text{H} + k_2)}{k_2 k_3 (R - C_4)} \\ - \frac{dC_4}{dH} \left( 6.909H + \frac{2.303 Rk_4 H (\text{H}^2 + 2k_2 \text{H} + 3k_2 k_3)}{k_2 k_3 (R - C_4)^2} \right)$$

Accurate values for  $C_4$  and its derivative can be calculated through only a part of the pH range, since for the one a difference, and for the other a ratio, of two quantities having a very small proportionate difference are required. This also applies to other ranges when equations are used based on the concentration of some other citrate ion. Consequently, equations for each ionic concentration have been derived and also the corresponding expressions for the buffer index. Each set of equations has been applied in that portion of the pH range in which the concentration of the ionic form involved is changing rapidly with pH. It has been found more accurate to calculate ratios of increments of concentrations rather than derivatives. Below are given the expressions for each concentration and the corresponding equation for the buffer index.

$$(24) \quad C_1 = \frac{k_1 k_2 k_3}{2w} [\sqrt{(R - S + wK)^2 + 4SwK} - (R - S + wK)]$$

$$(25) \quad \frac{dB}{dpH} = -\frac{4.606 C_2 H(k_2 + H)}{k_2 k_3} - \frac{dC_1}{dH} \left( \frac{6.909 k_4 R H}{(C_2 - k_4)^2} - \frac{2.303 H(3k_2 k_3 + 2k_2 H + H^2)}{k_2 k_3} \right)$$

$$(26) \quad C_2 = \frac{k_1 k_2 H}{2w} [\sqrt{(R - S + wK)^2 + 4SwK} - (R - S + wK)]$$

$$(27) \quad \frac{dB}{dpH} = \frac{6.909 k_3 k_4 R C_2 H}{(k_3 C_2 + k_4 H)^2} + \frac{2.303 C_2(3k_2 k_3 - H^2)}{k_2 H} - \frac{dC_2}{dH} \left( \frac{6.909 k_3 k_4 R H^2}{(k_3 C_2 + k_4 H)^2} + \frac{2.303(3k_2 k_3 + 2k_2 H + H^2)}{k_2} \right)$$

$$(28) \quad C_1 = \frac{k_1 H^2}{2w} [\sqrt{(R - S + wK)^2 + 4SwK} - (R - S + wK)]$$

$$(29) \quad \frac{dB}{dpH} = \frac{13.818 k_2 k_3 k_4 R C_1 H^2}{(k_2 k_3 C_1 + k_4 H^2)^2} + \frac{4.606 C_1(3k_2 k_3 + k_2 H)}{H^2} - \frac{dC_1}{dH} \left( \frac{6.909 k_2 k_3 k_4 R H^2}{(k_2 k_3 C_1 + k_4 H^2)^2} + \frac{2.303(3k_2 k_3 + 2k_2 H + H^2)}{H} \right)$$

In the exact application of these equations, it would be necessary to alter the values of the dissociation constants concerned by a correction calculated from the ionic strength of the solution and a coefficient depending for its value on the valence of the ionic form involved. However, since Simms (5) has shown that citric acid does not obey the Debye-Hückel equation, so far as the values of  $k_2$  and  $k_3$  are concerned, and since values applicable here are not available, the classical dissociation constants have been used in calculating values for points on the buffer curves and allowance for ionic attraction corrections has been made in the discussion.

Figs. 4 and 5 show buffer curves calculated for 0.05 M tricalcium citrate and 0.05 M dicalcium citrate, respectively. The values used for dissociation constants were,  $k_1 = 8.91 \times 10^{-4}$ ,  $k_2 = 1.76 \times 10^{-5}$ ,  $k_3 = 3.89 \times 10^{-7}$  (1), and  $k_4 = 6.03 \times 10^{-4}$  (3), and the calculations were based on Equations 16, 17, and 23 to 29.

It is well known that, in the plots of the more usual types of buffer action, maxima for  $dB/dpH$  occur at pH values numerically equal to the practical pK values of the weak acid involved. When this equality exists, concentrations of two forms of the

acid are equal, or, graphically, the ascending curve of the concentration of one form is crossing the descending curve of the concentration of another. In plots of the buffer action of the

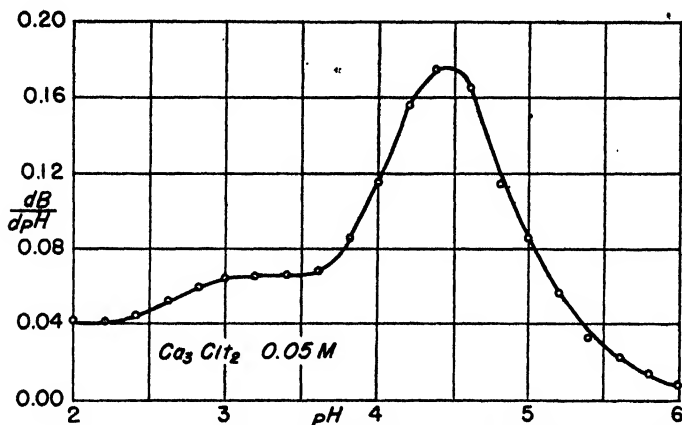


FIG. 4. Theoretical curve for buffering by 0.05 M tricalcium citrate

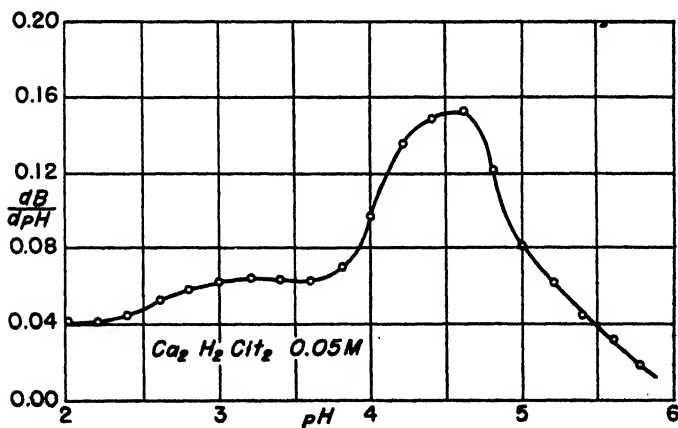


FIG. 5. Theoretical curve for buffering by 0.05 M dicalcium citrate

usual weak polybasic acids with widely separated pK values, these maxima are of practically the same value and the concentrations of each of the two forms corresponding to the intersection of the

concentration curve are very nearly one-half of the total concentration of the acid.

If the concentrations of the five forms of citric acid present in a 0.05 M solution of calcium citrate are plotted against pH, as shown for tricalcium citrate in Fig. 6, the pH values at the intersections are the same as when calcium is absent, but the concentrations at some of these intersections will be considerably less than normal because of the presence of a large proportion of the citrate in the calcium citrate complex. The intersection of the  $C_2$  and  $C_3$  curves for 0.05 M  $\text{Ca}_3\text{Cit}_2$  is at about 0.01 the con-

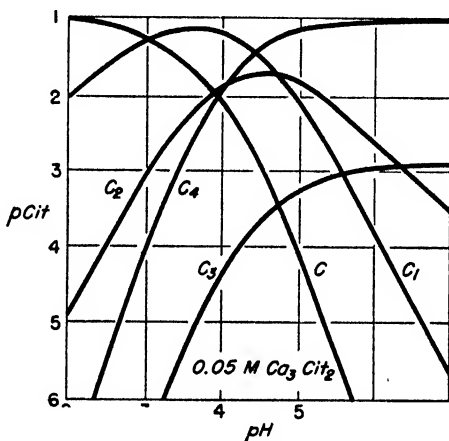


FIG. 6. Ionic concentration curves for 0.05 M tricalcium citrate.  $p\text{Cit}$  is the logarithm of the reciprocal of the concentration of each species of citrate plotted.

centration of these forms in a corresponding calcium-free solution, which indicates that practically no hump may be expected in the region of pH 6.4.  $C_3$  and  $C_4$  do not intersect at all, and  $C_3$  does not increase beyond about 0.01 the higher values of  $C_4$ . The point of intersection of the  $C$  and  $C_1$  curves is practically unchanged. There are four intersections in the region between pH 3.8 and 4.8; namely,  $C$ - $C_2$ ,  $C_2$ - $C_4$ ,  $C_1$ - $C_4$ , and  $C_1$ - $C_2$ . The  $C_1$ - $C_4$  intersection is at the highest concentration of the three, and, since in this shift 2 hydrogen ions are involved per molecule, the theoretical maximum at this point should be doubly high. The reinforcing action of the other intersections in this region, particu-

larly of  $C_1$ - $C_2$ , increases the total height of the peak, as shown in the curves constructed from the buffer equation.

Simms (5) found the Debye-Hückel coefficient for  $pK_2$  for citric acid in the presence of  $MgCl_2$  to be 6.6. If the assumption is made that the effects of  $CaCl_2$  are of the same order, the corrected value of  $pK_2$  would be approximately 4.1 for the solutions being considered. Analogously, the peak in the classical curve should be shifted from pH 4.45 to 3.75. In the curves derived from experiment, a peak occurs at pH 3.7. The pH values at which the  $C_4$  curve intersects the other curves are slightly higher for dicalcium citrate than for tricalcium citrate solutions and  $C_2$  rises to one-tenth the value of  $C_4$  at approximately pH 5.0.

Although this discussion is based to a certain extent on assumptions and is therefore open to attack on that basis, the assumptions seem reasonable and lead to a conclusion that the Hastings-McLean idea of the mechanism of ionization in solutions containing calcium and citrate is correct. The author feels that, in respect to their work, this paper merely adds to evidence already reasonably sufficient.

In attempting to apply the above data to milk, it must be remembered that the total citrate of milk is approximately 0.01 M—one-tenth the concentration used in these experiments—and that the concentration of calcium in the serum of milk is of the same order of magnitude. Furthermore, the square root of the ionic strength of milk is approximately one-third of that of these solutions. Consideration of these facts indicates that, if the calcium and citrate of milk serum functioned as an independent system, its maximum buffer intensity would be about 0.010 and its buffer action would be exerted principally in the region of pH 4.2 to 4.5. It is in this region that the heterogeneous buffering caused by calcium phosphate is most intense and predominant, as shown in the preceding paper of this series, and, because of the diminished quantity of ionic calcium that can be present above pH 4.4 in equilibrium with phosphate ions, part of the citrate buffering will be spread toward higher pH values, tending to resemble that of calcium-free citrate solutions. But it will still be exerted largely in a region where other buffers are predominant—calcium phosphate and casein. Therefore, it may be concluded that this work has shown that the buffer action of citrate in milk is exerted



over a much narrower range than would be expected from a consideration of the buffer curve of a calcium-free citrate solution and that this range is essentially that in which calcium phosphate and casein buffer most intensively. Its participation is undoubtedly of little importance from the buffer standpoint in the complex equilibrium involving simultaneously calcium, casein, and the various forms of phosphate and citrate, so that only its direct participation need be considered in formulating the mechanism of the buffer action of milk.

#### SUMMARY

Equations describing the buffer action of calcium citrate have been derived and curves based on these equations compared with curves constructed from potentiometric titrations of calcium citrate solutions. The results support the Hastings-McLean idea of the mechanism of ionization of calcium citrate in solution.

Application of these results to milk equilibria indicates that the buffer action of citrates in milk is exerted principally in the range in which phosphates and casein buffer most intensely and is of slight moment compared with the effects of these other buffer substances.

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# THE EFFECT OF GROWTH ON THE DISTRIBUTION OF WATER AND ELECTROLYTES IN BRAIN, LIVER, AND MUSCLE\*

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Aside from lipids and the supporting framework, such as bone and connective tissue, the animal body may be considered as consisting essentially of extracellular and intracellular water together with the respective organic and inorganic solutes contained in each compartment. The purpose of this paper is to present data which will permit a qualitative and roughly quantitative estimation of the differences in these two compartments in some of the tissues of cats of various ages. It is hoped that by this means a more adequate conception of the nature of the physical growth of these tissues may be obtained.

## *Procedure*

Because of the difficulty of verifying the exact ages of the animals, the weight was used as the index of growth. The animals varied in weight from approximately 300 to 2500 gm. Roughly, this range covers an age period of from 4 weeks to about 6 months or more. Some of the animals were presumably full grown adult cats whose ages could not be estimated. They were received at the laboratory in good condition at least 1 week before the analyses were carried out. During this period they were fed a diet of canned meat and milk. No food was given the day of the experiment; water was allowed *ad libitum*.

The animals were anesthetized with ether and killed by exsanguination through a cardiac puncture. An adequate quantity of blood was collected under mineral oil, allowed to clot, and

\* This work was aided by a grant from the Fluid Research Fund of the School of Medicine.

the serum removed. The entire liver and adequate amounts of muscle (from all extremities) were placed in previously weighed, stoppered bottles. The brain, including the hemispheres, cerebellum, and most of the midbrain, was carefully delivered into a weighed, stoppered bottle after all visible particles of bone were wiped away.

### *Calculations and Methods*

The calculations employed for estimating intracellular and extracellular water and the concentration of intracellular nitrogen, potassium, and phosphorus are based on the premise that all tissue chloride is extracellular. Essentially similar results could be obtained if one assumed that sodium is exclusively extracellular. The formulæ, their derivation, and discussion of their validity are given in a previous publication (1). The chemical methods for the analysis of blood and tissues are described elsewhere (2).

### *Results*

The results of the chemical analyses of brain, muscle, and liver are summarized in Table I. In order to conserve space only the average concentrations of water, nitrogen, chloride, sodium, potassium, phosphorus, and fat are given. The values following the averages are the standard errors, calculated from the formula  $\sigma/\sqrt{n-1}$  where  $\sigma$  is the standard deviation and  $n$ , the number of analyses. For purposes of simplifying the discussion, the animals are divided into Groups A and B. Group A is made up of the younger animals, ranging in weight from about 300 to 800 gm., and averaging 560 gm.; Group B, the older animals, ranging from 800 to about 2500 gm., and averaging 1600 gm. Water, nitrogen, and fat are expressed as gm.; the remaining values as millimoles per kilo of whole tissue. Unfortunately, because of insufficient material, especially in the younger group, fat analyses could not be carried out on every tissue. For this reason, it was not possible to express the results in terms of fat-free material. However, an approximate idea of the magnitude of the tissue lipids in each group may be obtained from the given values which represent the averages of somewhat more than half the tissues in each group.

In order to present the changes accompanying growth the tissues of the older animals (Group B) will be compared with those of the younger ones (Group A). In the brain the following changes are noted in Group B: (1) a decrease in total water, chloride, and sodium; (2) an increase in nitrogen, phosphorus, and fat. In muscle, the older group shows (1) a decrease in chloride and sodium, (2) an increase in nitrogen, potassium, and phosphorus, and (3) no significant change in water or fat. In the liver, the older group shows (1) an increase in fat and decrease in water,

TABLE I

*Concentrations per Kilo of Tissue of Water, Nitrogen, Chloride, Sodium, Potassium, Phosphorus, and Fat in Brain, Muscle, and Liver of Young and Older Animals*

Group A includes animals up to 800 gm. in weight; Group B, animals from 800 to 2500 gm.

Group	No. of rats	Water	Nitrogen	Chloride	Sodium	Potassium	Phosphorus	Fat
Brain								
		gm.	gm.	mM	mM	mM	mM	gm.
A	9	846± 5.0	14.7±0.3	43.2±1.4	55.1±1.4	87.0±1.6	76.9±3.7	30
B	8	808± 4.0	16.8±0.2	39.0±1.0	52.5±1.2	88.0±2.2	96.3±0.5	60
Muscle								
A	11	785± 8.0	24.7±0.6	28.2±1.3	36.8±2.0	80.0±1.8	59.0±2.4	20
B	9	770± 3.0	29.1±0.6	15.1±1.2	23.3±1.6	90.4±2.4	66.4±1.5	25
Liver								
A	11	730± 8.0	23.7±3.7	31.0±1.4	38.6±2.0	80.1±2.2	75.6±3.5	25
B	9	707±10.0	27.4±3.4	27.7±1.3	33.3±1.7	73.7±1.7	90.3±2.5	60

(2) an increase in nitrogen and phosphorus, and (3) a decrease in chloride, sodium, and potassium. It will be noted that there is a much greater scattering of results in the liver than in the other tissues, as evidenced by larger values for the standard errors of most of the determinations. Moreover, some of the changes, such as the water and nitrogen, can hardly be considered significant, the differences of the means being less than 2 times the standard error of the differences.

Table II gives the average proportions of extracellular and

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intracellular water, and the average concentrations of intracellular nitrogen, phosphorus, and potassium, in brain, muscle, and liver of the two groups representing, respectively, the younger and older animals. These results were obtained by the application of the formulæ and calculations described in the article by Harrison, Darrow, and Yannet (1) to the data, a summary of which was presented in Table I. The standard error of each average value is also included. Columns 3, 4, and 5 give the total,

TABLE II

*Distribution of Water and Electrolytes in Brain, Muscle, and Liver of Young Animals*

Group A includes animals weighing up to 800 gm.; Group B, animals weighing from 800 to 2500 gm.

Group	No. of cats	Total water	Extra-cellular water	Intra-cellular water	Intra-cellular N	Intra-cellular K	Intra-cellular P	Fat
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Brain								
		per cent	per cent	per cent	gm. per l.	mM per l.	mM per l.	per cent
A	9	84.6±0.5	33.9±1.1	50.7±1.0	29.1±0.7	167±3.5	160±5.0	3.0
B	8	80.8±0.4	29.8±0.6	51.0±0.9	33.1±0.9	166±4.0	189±4.0	6.0
Muscle								
A	11	78.5±0.8	21.8±1.1	56.7±1.1	43.6±1.2	139±3.0	104±3.2	2.0
B	9	77.0±0.3	11.7±0.8	65.3±0.8	44.6±0.5	137±2.2	102±1.5	2.5
Liver								
A	11	73.0±0.8	24.2±1.2	48.8±1.3	47.6±2.0	161±4.0	155±6.5	2.5
B	9	70.7±1.0	21.2±1.0	49.4±1.2	55.7±2.0	145±2.8	184±5.0	6.0

extracellular, and intracellular water of each tissue expressed as per cent of tissue weight. Columns 6, 7, and 8 give the concentrations of nitrogen, potassium, and phosphorus per liter of intracellular water. Nitrogen is expressed as gm. and potassium and phosphorus as millimoles. Column 9 gives the average fat content of the tissues examined expressed as per cent of tissue weight.

The distribution of water and electrolytes in the brains of the older group (B) differ from that in the younger group (A) in the

following manner: (1) a decrease in the percentage of extracellular water, (2) an increase in the concentration of intracellular nitrogen and phosphorus. There were no changes in the concentration of intracellular potassium, or in the proportion of intracellular water.

In the muscle, the older group shows (1) a decrease in the percentage of extracellular water, (2) an increase in the percentage of

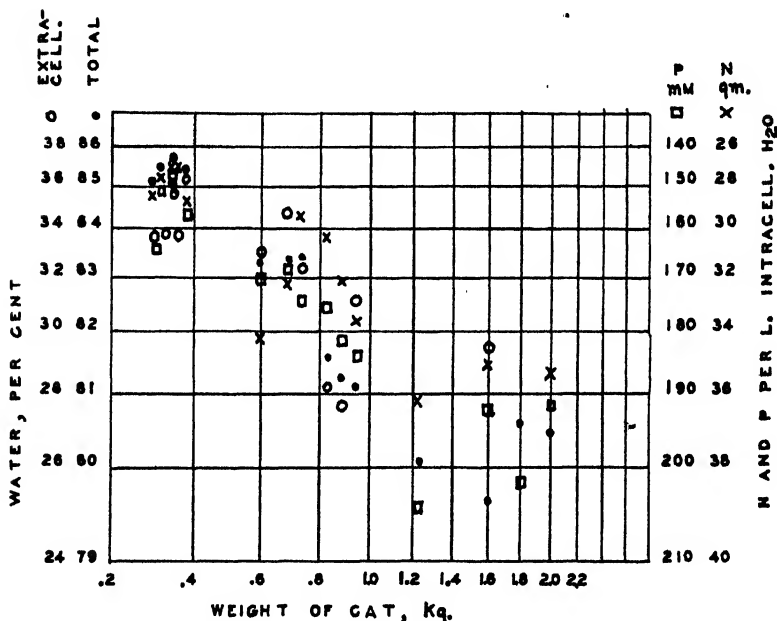


FIG. 1. The relationship of the distribution of water and the concentration of intracellular nitrogen and phosphorus of the brain to the growth of the body as a whole.

intracellular water, (3) no significant changes in the concentration of intracellular nitrogen, potassium, and phosphorus.

In the liver, the older group shows (1) a decrease in the percentage of extracellular water, (2) no change in the percentage of intracellular water, (3) an increase in the concentrations of intracellular nitrogen and phosphorus, and (4) a decrease in the concentration of intracellular potassium.

Figs. 1 and 2 were prepared in order to demonstrate the progres-

sive nature of the changes involved in the process of physical growth in the brain and muscle. They also serve to present graphically the relationship of the changes to each other or to some common factor. In Fig. 1 the weights of the animals are represented on the abscissa, while on the ordinate a number of factors for the brain are represented; namely, per cent of total water, per cent of extracellular water, and the concentrations of intracellular nitrogen and phosphorus. Four different scales

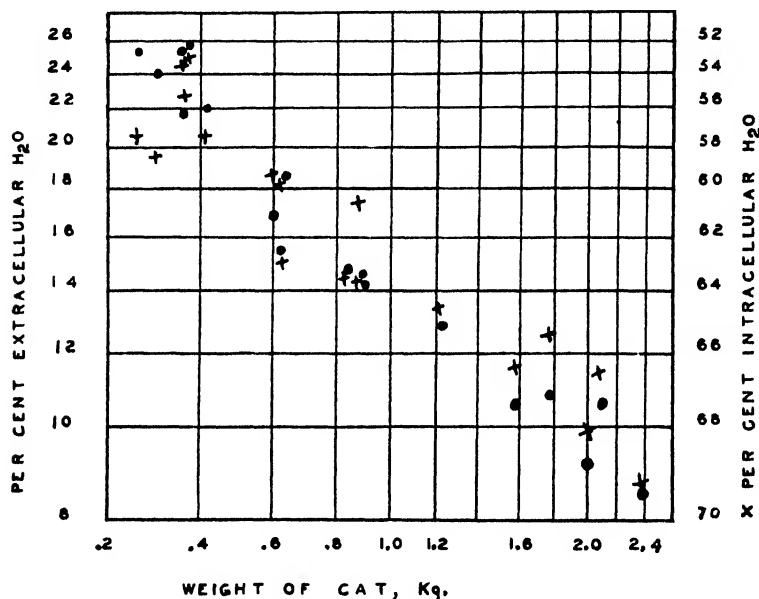


Fig. 2. The relationship of the distribution of water in muscle to the growth of the body as a whole.

were chosen so that the maximum variations of each factor occupy about the same space on Fig. 1. Both the weights of the animals and the various concentrations are plotted on a logarithmic scale as suggested by Huxley (3). As might be expected from the nature of the calculations employed, a rather wide scattering of the individual values is obtained. However, the tendency for all the values to fall roughly into an area bounded by two lines of similar slope is striking. This suggests that the changes in composition with respect to the proportions of total water and

extracellular water and concentrations of intracellular nitrogen and phosphorus are probably closely associated with a common factor which, as will be pointed out later, is the increase in lipid of the brain.

Fig. 2 is prepared to show the relationship between the changes in the proportions of extracellular and intracellular water in muscle, resulting from growth.\* The ordinate represents extracellular and intracellular water expressed as per cent of tissue weight. The abscissa represents the weight of the animal. All values are similarly plotted on a logarithmic scale. The straight line relationship and the similar slope of the lines representing the two fluid compartments of muscle can be noted. In other words the growth of muscle is accompanied by an increase in the proportion of intracellular water and a decrease in the proportion of extracellular water.

When a similar chart was prepared from the results obtained from the liver analyses, no correlation was noted between the distribution of water or electrolytes and the weight of the animals. Hence the changes in the liver are presumably not related to growth in any regular, progressive manner.

#### DISCUSSION

The relationship between the growth of certain organs and the growth of the body as a whole has been called by Huxley (3) "the constant differential growth ratio," and has been defined by the equation  $y = kx^p$ , where  $k$  and  $p$  are constants. Expressed in logarithmic form the equation becomes,  $\log y = p \log x + \log k$ . Hamilton (4) has recently described this type of relationship between total body water and body weight, using the data of Iob and Swanson on the chemical analyses of human fetuses. It is apparent that Figs. 1 and 2 describe changes in the chemical composition of brain and muscle which show a relationship to growth of the body according to "the constant differential growth ratio." One may surmise, therefore, that the changes in chemical composition of these tissues are associated with growth of definite anatomical structures.

In the growth of tissues, chemical composition may be altered in two ways: first, by increase in size, where the extra tissue is of different chemical composition than that already present, and



second, by changes in the make-up of the original tissue. Recognizing the complex nature of the effects of tissue growth on chemical composition, we may visualize the changes by calculating the proportions of various constituents in the "increment of growth." The increment of growth may be defined, for our purposes, as the tissue which, when added to that of the young animal, yields a tissue of the composition of the older animal. The calculation requires the initial and final concentrations and weights. The formula for the calculation is

$$P_i = \frac{BP_b - AP_a}{B - A}$$

where  $P_i$ ,  $P_b$ , and  $P_a$  refer to the per cent composition of the "increment," the larger tissues, and smaller tissues respectively and  $B$  and  $A$  are the weights of the larger and smaller tissues respectively. The average weight of the brains in Group A was 16 gm. and of Group B, 23 gm. Exact knowledge of the actual increase in size of muscle and liver in the cats is not available. In calculating the increment of growth in these tissues, we have assumed a 3-fold increase in both cases. This is based on the fact that human livers increase approximately directly with the weight of the body (Scammon (5)), and a similar increase may be expected from his data on muscles.

The composition of the "increment of growth" of brain, muscle, and liver, together with those of the tissues of the two groups, is given in Table III. In the brain, increment of growth shows a larger per cent of fat, a smaller per cent of extracellular water, with little change in intracellular water or fat-free solids. In other words, the growth of brain was attained by expanding all elements but with a laying down of lipids which seem in part to occupy space that one might otherwise expect to be taken up by extracellular fluid. The increased concentration of intracellular nitrogen and phosphorus (see Table II) is largely dependent on increase in the concentration of lipid rather than any change in composition of non-lipid cytoplasm. Recent analyses in this laboratory indicate that brain lipid of cats contains about 2.2 gm. of nitrogen and 45 millimoles of phosphorus per 100 gm. With use of these values, the changes in concentration of intracellular nitrogen and phosphorus are entirely accounted for by the increase in lipids.

Histologically the brain of older animals differs from that of younger animals in the following ways: (1) there is an increase in the size of the individual nerve cells without any striking change in the relative magnitude of the extracellular space; (2) there is an increase in size and number of dendrites and glial processes; (3) there is an increase in the white matter with its high content of lipid; (4) there is practically no change in the number of cells.

The increase in the size of the nerve cells and in the number and size of dendrites and perhaps glial processes accounts for the expansion of the intracellular fluid. The lipid of the white matter accounts for the increase in lipids as well as nitrogen and phosphorus. As indicated by Fig. 1 the changes in composition of the brain are related in a fairly regular manner to the increase in

TABLE III  
*Percentage Distribution of Water, Fat, and Fat-Free Solids in "Increment of Growth" in Brain, Liver, and Muscle*

		Extracellular water	Intracellular water	Fat	Fat-free solids
Brain	Group A	33.9	50.7	3.0	12.4
	" B	29.8	51.0	6.0	13.2
	Increment	20.5	51.7	12.8	15.0
Liver	Group A	24.2	48.8	2.5	24.5
	" B	21.2	49.4	6.0	23.4
	Increment	19.7	49.7	7.8	22.8
Muscle	Group A	21.8	56.7	2.0	19.5
	" B	11.7	65.3	2.5	20.5
	Increment	6.7	69.6	2.7	21.0

weight of the animal. In other words, the changes in composition of the brain are related to the growth of certain structures of the brain which are, in turn, related to growth of the body as a whole.

The composition of the "increment of growth" in the liver shows a larger per cent of fat, a smaller per cent of extracellular fluid, together with little change in intracellular water and fat-free solids. In other words, as in the brain, growth of the liver was attained by expanding all elements, but with an increased concentration of lipids and a decrease in extracellular fluid. The increase in concentration of phosphorus and nitrogen (see Table II) is probably related to the increase in liver lipid rather than changes in non-lipid cytoplasm. At present the decrease in the concentration of intracellular potassium in the livers of older

animals cannot be explained. However, evidence is accumulating that, although concentration of total base in extracellular fluid determines the hydration of cells, the concentration and hence the ionic activity of intracellular potassium may vary independently of the concentration of extracellular base (2).

Histologically the liver of young animals may be considered a miniature of that of the older animals, since its growth involves reduplication of liver lobules. It will be recalled that no regular relationship such as was shown in Figs. 1 and 2 for the brain and muscle was demonstrable between the changes in composition of the liver and the growth of the body as a whole. This indicates that the changes in composition of the liver reflect alteration in the metabolic activity or state of nutrition rather than structural changes associated with growth. Thus, although the changes in liver nitrogen and phosphorus are dependent on the increase in liver lipid, the latter is related to the state of nutrition rather than growth of the animal. Although the cats in the two groups were handled in the same way, the effect on the state of nutrition was different, since short periods of fasting produce more rapid changes in young animals than older ones, and the younger cats do not thrive in laboratories.

In the muscle, the composition of the "increment of growth" shows that muscle grows chiefly by an expansion of the intracellular phase with surprisingly little increase in the extracellular fluid. Histologically the muscle of older animals shows an increase in the size of the individual muscle fibers with a relative decrease in the size of the interstitial spaces. In Fig. 2 the increase in the proportion of intracellular water and the decrease in the proportion of extracellular water were shown to be directly related to the growth of the animal as a whole. In other words, since the changes in composition of the muscle are brought about by the increase in size of muscle fibers, the growth of muscle fibers is related to the growth of the animal as a whole according to the constant differential growth ratio. Changes of a similar nature during the growth of muscle have been described by Kerpel-Fronius (6) in dogs, rabbits, and humans.

#### SUMMARY

The effect of growth on the chemical composition of brain, liver, and muscle was studied in cats of different ages.

In the brain, growth is associated with a decrease in the concentration of water, sodium, and chloride, and an increase in the concentration of fat, phosphorus, and nitrogen. These changes are related to the growth of the body as a whole according to Huxley's "constant differential growth ratio." Anatomically, the changes are brought about chiefly by the deposition of lipid in the myelin sheaths, with an associated decrease in the proportion of extracellular fluid.

In muscle, growth is associated with a decrease in the concentration of sodium and chloride, and an increase in the concentration of nitrogen, phosphorus, and potassium. There is little change in the percentage of total water. These changes in chemical composition represent a change in the distribution of water between the intracellular and extracellular compartments resulting from the growth of the muscle fibers. The relationship of the increase in the percentage of intracellular fluid and the decrease in percentage of extracellular fluid, with the growth of the body as a whole, also follows the "constant differential growth ratio."

The livers of the older animals show an increased concentration of lipids, phosphorus, and nitrogen, and a decreased concentration of sodium, chloride, and water. These changes are all apparently associated with the deposition of lipid and are related to the nutritional status of the animals rather than to growth.

Except for a decrease in the concentration of intracellular potassium in the livers of the older animals, no changes are demonstrated in the concentration of non-lipid nitrogen, non-lipoid phosphorus, or potassium in the intracellular fluid of the tissues studied.

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## THE ACTION OF *p*-AMINOPHENOL ON THE XANTHINE OXIDASE OF LIVER

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The quinimine form of *p*-aminophenol inhibits the oxygen uptake of tissue suspensions and in very small concentrations inhibits specifically the oxidation of hypoxanthine and xanthine to uric acid by the xanthine oxidase of liver (1). Closely related compounds such as *o*-aminophenol, *p*-phenylenediamine, and quinone, although they inhibit to different degrees the oxygen uptake of tissues, have no effect on the xanthine oxidase. Because none of the other common dehydrogenases is affected by the quinimine and because the xanthine oxidase is inhibited by it in concentrations as low as 0.00001 M, it was of interest to study the mechanism of the inhibition more closely.

### EXPERIMENTAL

For a quantitative study of an inhibition of this type the use of the purest form of the enzyme is desirable. The xanthine oxidase of milk was therefore prepared according to the method of Dixon and Thurlow (2). Unfortunately, the quinimine had a very variable effect on the milk oxidase. In some preparations a 50 per cent inhibition could be obtained but in most preparations no inhibition occurred under conditions which caused 100 per cent inhibition of the liver oxidase. There were three possible reasons for this discrepancy. Either the liver changed the quinimine, or the quinimine acted on something in the liver which in turn inhibited the oxidase or there was something in milk that prevented the inhibition from taking place. The following experiment indicates that milk contains a substance which inactivates the quinimine. A small amount of milk oxidase was added to liver

and the quinimine inhibition compared to that obtained in the liver alone. The results showed that whereas the oxidation of added xanthine was completely inhibited in the control, the presence of milk oxidase abolished the inhibition and xanthine was oxidized at a rate which showed that both the liver and milk oxidase were catalyzing its oxidation.

For the study of the inhibition the xanthine oxidase of rat liver was therefore used. It was necessary to determine first whether the other metabolic activities of the liver had any effect on the xanthine oxidase inhibition. A rat liver was ground with sand in 10 cc. of 0.05 M phosphate buffer at pH 6.7. A part of this was used immediately, a part was put in the ice box for 24 hours, and a part was dialyzed for 24 hours against distilled water. The fresh liver had a large oxygen uptake which after 24 hours had been reduced to a quarter and after dialysis disappeared completely.

TABLE I

*Effect of Different Concentrations of Quinimine on Oxygen Uptake of Rat Liver and on Xanthine Oxidase at pH 6.7*

Concentration of quinimine $\times 10^{-3}$ M.....	10	5	2.5	1.6	1.2
	per cent	per cent	per cent	per cent	per cent
Inhibition of xanthine oxidase .....	100	94	90	68	45
" " liver .....	61	45	20	18	16

The xanthine oxidase activity of all three preparations was the same. In each case 0.00005 M quinimine completely inhibited the oxidation of xanthine. The percentage inhibition of the oxygen uptake of fresh liver by different concentrations of quinimine was then compared with the percentage inhibition of xanthine oxidase. As the concentration of quinimine was decreased, the liver inhibition decreased more rapidly than the xanthine oxidase inhibition until at a concentration of 0.00001 M the liver was only 16 per cent inhibited but the xanthine oxidase was still 45 per cent inhibited. These results are shown in Table I. In each case the percentage inhibition remained constant during the experiment.

The effect of pH on the inhibition of liver by quinimine was compared with its effect on the inhibition of the xanthine oxidase preparation. As the liver suspension was made more alkaline, the inhibition of the oxygen uptake decreased from 66 per cent

at pH 6.7 (with 0.0001 M quinimine) to 22 per cent at pH 7.8. Correspondingly the xanthine oxidase inhibition decreased from 100 to 69 per cent. This is shown in Table II. There are two possible reasons for the decreasing inhibition with increasing pH. Either there is a dissociation of the quinimine-enzyme complex or the drug is inactivated or destroyed in alkaline solutions. If the latter alternative were correct, then there should be a progressive decrease of the inhibition with time when the drug is used at pH 7.8. The inhibition, however, of both the liver and the xanthine oxidase remains constant for a period of at least 3 hours, which proves that no progressive destruction of the quinimine was taking place. The pH effect must be caused by the dissociation of the drug enzyme complex in alkaline solutions and this is also indicated by the fact that it is possible to obtain complete

TABLE II

*Effect of Hydrogen Ion Concentration on Inhibition of Oxygen Uptake of Rat Liver and of Xanthine Oxidase by 0.0001 M Quinimine*

pH.....	6.7	7.0	7.3	7.5	7.8
	per cent	per cent	per cent	per cent	per cent
Inhibition of xanthine oxidase.....	100	93	84	77	68
"    "    liver.....	66	58	48	43	22

inhibition of the xanthine oxidase at pH 7.8 with concentrations of quinimine varying between 0.0003 M and 0.0002 M.

The rate of oxidation of hypoxanthine at pH 7.3 to 7.8 was about 90 to 100 c.mm. per hour and at pH 6.7 to 7.3 it was 80 to 90 c.mm. per hour. There was therefore no correlation between rate of oxidation and percentage inhibition. Moreover, the concentration of xanthine or hypoxanthine present had no effect on the inhibition; at pH 6.7 complete inhibition of the xanthine oxidation was obtained when amounts varying from 0.25 to 3.0 mg. of xanthine were being oxidized.

*p*-Aminophenol autoxidizes in solution and the rate increases with the pH. It is in equilibrium with quinimine, and its potential is close to that of the quinhydrone system. Theoretically, then, it should not matter whether the reduced or the quinimine form is added to the liver or the xanthine oxidase. Reduction or



oxidation of the quinimine-*p*-aminophenol system should occur until it is in equilibrium with any other systems that happen to be present in the preparation. In the presence of liver, however, whether it is freshly prepared or dialyzed, this does not occur. Addition of *p*-aminophenol does not inhibit the oxygen uptake of the liver at all at pH 7.8, whereas the addition of the quinimine<sup>1</sup> inhibits it 20 per cent. It is at this pH that autoxidation should occur most readily. At pH 6.7 the reduced form inhibits the liver only 30 per cent while the quinimine inhibits 60 per cent. The xanthine oxidase is not inhibited at all at either pH by the reduced form. The probable explanation for the non-interchangeability of the two forms in the presence of liver is that the receptors in the liver immediately combine with the drug so that it can no longer act as a reversible oxidation-reduction system.

That some such combination of the quinimine with liver occurs is indicated by the following experiment. In order to prove the reversibility of the inhibition of the xanthine oxidase a series of Warburg vessels was put up in the usual way with hypoxanthine, freshly prepared liver at pH 6.7, and 0.0001 *M* quinimine. As shown by Curve 6 in Fig. 1, the inhibition by the drug was almost complete. At intervals a control vessel and one containing hypoxanthine were taken off and the contents transferred to Thunberg tubes. These were then evacuated and incubated at 37°. Even though the drug when added to liver and shaken in air is not oxidized or reduced, the results as shown in Fig. 1 indicate that the quinimine is reduced *in vacuo* by the liver. Once the reduction had taken place, the solutions were placed again in the Warburg vessels and the oxidation of hypoxanthine which before the reduction was almost completely inhibited now proceeded at the same rate as the control with no drug added. But as Curve 5 shows, it requires a reduction period of at least an hour before complete recovery takes place. The vessels which Curve 5 represents were taken off after the inhibition had been allowed to run only half an hour. They remained *in vacuo* for half an hour and the subsequent recovery was not complete, indicating that there was still quinimine present. For Curve 3 the inhibition was allowed

<sup>1</sup> This is prepared by warming a solution of *p*-aminophenol and then allowing it to stand at room temperature for half an hour. The *p*-aminophenol was recrystallized from alcohol and water.

to run for 1 hour before reduction but the reduction was carried on for an hour. This time, despite the longer period of inhibition,

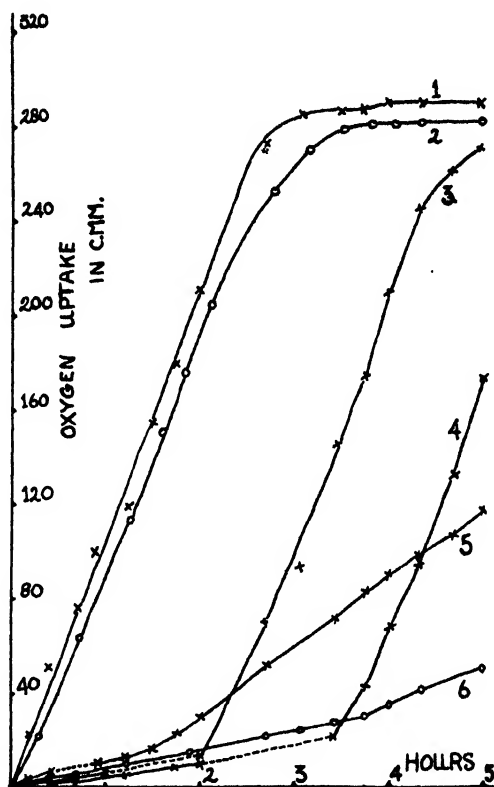


FIG. 1. The oxidation of 2 mg. of hypoxanthine by rat liver at pH 6.7. Curve 1 control, Curve 2 with 0.0001 M *p*-aminophenol, Curve 3 with 0.0001 M quinimine after 1 hour's incubation anaerobically, Curve 4 same as Curve 3 after 1½ hours' incubation anaerobically, Curve 5 same as Curve 3 after ½ hour's incubation anaerobically, Curve 6 same as Curve 3 except that there was no anaerobic incubation. The dotted lines are extrapolations representing the time of anaerobic incubation. Each curve represents the oxygen uptake of liver plus 2 mg. of hypoxanthine minus the uptake of the liver alone.

the recovery was complete, as shown by the slope of the curve. For Curve 4 the inhibition ran for 2 hours, and complete recovery occurred after 1½ hours reduction. This experiment shows that

liver which will reduce 200 micrograms of methylene blue in 3 to 5 minutes under these conditions takes over half an hour to reduce 20 micrograms of quinimine, although the latter belongs to an oxidation-reduction system with a higher potential than the methylene blue system. The indications therefore are that the drug is not in solution but closely bound to the receptors in the liver. Fig. 1 also shows that the reduced form is without effect (Curve 2) and that complete reversibility is possible no matter how long the inhibition has run. Further evidence for the combination of the drug with liver receptors is the fact that a 4 hour dialysis at pH 6.7 of liver to which quinimine had been added does not restore the oxidation of xanthine. This experiment was carried out by placing liver and quinimine in a dialyzing bag and dialyzing against a buffer in a flask which was shaken in air at 37°. A control without quinimine was similarly shaken and showed the usual xanthine oxidase activity.

Because quinimine had no effect on any of the other dehydrogenases tried (1), it is possible that its action on the xanthine oxidase is due to some peculiarity of the enzyme structure. It was therefore of interest to try its effect on guanase and uricase, enzymes which attack substrates the chemical structure of which is closely related to xanthine. The rat liver contains an active guanase. Its activity could be measured by the ammonia liberated and also by the rate of oxygen uptake due to the xanthine formed from the deamination of the guanine. This latter method also served to check the activity of the added quinimine, for in its presence the oxidation of xanthine was completely suppressed. Warburg vessels were set up in the usual way with either freshly prepared or dialyzed rat liver suspension and guanine. When the theoretical amount of xanthine had been formed from the guanine as shown by the oxygen uptake of the control, the amount of free ammonia was estimated by a vacuum distillation method. A large number of experiments were carried out and the results showed that guanase was inhibited from 30 to 50 per cent at pH 6.7 by the quinimine. Increasing the concentration of the drug did not increase the percentage inhibition nor did varying the relative concentrations of drug, guanine, and guanase. The pH effect was similar to that on the xanthine oxidase. In a typical

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experiment the inhibition was 48 per cent at pH 6.7 and 12 per cent at pH 7.8. These experiments also showed that quinimine had no effect on the ammonia production of the liver suspension alone. It has already been shown (1) that the drug has no effect on  $\text{CO}_2$  production. Adenase was not tried because no active source of the enzyme could be obtained.

In order to test the action of quinimine on uricase an acetone preparation from pig liver was made according to the method of Keilin and Hartree (3). Although the conditions were varied in all possible ways, the oxidation of uric acid by the enzyme was not affected at all by the drug even in concentrations of 0.001 M. The uricase preparation still had a small xanthine oxidase activity. This was completely inhibited by small concentrations of quinimine, showing that there was nothing in the preparation that interfered with the action of the drug.

### DISCUSSION

The evidence indicates that both the reduced and oxidized forms of *p*-aminophenol combine with some substance in the liver and once in such a combination are no longer readily oxidized or reduced. The nature of the substance in the liver is not known. *p*-Aminophenol is not a very reactive compound. Except for its oxidizing and reducing properties, it reacts readily only with aldehydes. Consequently addition of small amounts of aldehyde to liver completely protects the liver and the xanthine oxidase from the action of quinimine (1). The inhibitory effect of cyanide on xanthine oxidase as shown by Dixon and Keilin (4) is of interest in this connection. Its action differs in that the presence of the substrate protects the enzyme against cyanide but not against quinimine. The difference in the action of the oxidized and reduced *p*-aminophenol may be due to a different type of complex formation. Once the reduced complex is formed, shaking in oxygen at 37° for at least 4 hours does not oxidize a significant amount of it. The oxidized complex, however, can be reduced *in vacuo* after incubation at 37° for about 1 hour.

The fact that quinimine is effective in such small concentrations makes it a more powerful inhibitor of oxygen uptake *in vitro* than cyanide, arsenite, pyrophosphate, or urethane. It also shows

greater specificity of action, for xanthine oxidase is the only one of the common dehydrogenases that is inhibited, whereas the other drugs act on groups of dehydrogenases. It is possible that there are other systems that are inhibited; in fact, unless xanthine and hypoxanthine play some cyclic rôle in the cell, the inhibition of xanthine oxidase alone cannot account for a 60 per cent inhibition in the oxygen uptake of liver. But until this question is decided, quinimine is the most specific of the substances that act on the known oxidation systems. As already shown (1) it has no effect on the autoxidation of sulfhydryl compounds or on carbon dioxide or ammonia production.

Addition of quinimine to liver slices suspended in Ringer's solution caused only small inhibitions of the oxygen uptake. This indicates that the drug does not readily penetrate the cell membrane. It has been shown (5) that quinimine is formed when such antipyretics as acetanilide and acetophenetidin are taken. Possibly the effectiveness of these substances may be due to their ability to penetrate through the cell membrane and thereby allow the quinimine to be formed inside the cell.

#### SUMMARY

1. The quinimine and probably also the reduced form of *p*-aminophenol make non-dialyzable complexes with receptors in the rat liver. Only the quinimine is active in inhibiting the oxygen uptake of the liver and the oxidation of hypoxanthine and xanthine by the xanthine oxidase.

2. The inhibition of the xanthine oxidase by the quinimine is reversible if the mixture is incubated anaerobically for an hour at 37°. The reduced *p*-aminophenol shaken in air at 37° with liver is not oxidized to the quinimine.

3. Varying the concentration of the quinimine affects differently the inhibition of the liver uptake and the xanthine oxidase. The pH effect on the inhibition is different in both cases also.

4. The xanthine oxidase of milk is usually not affected by the quinimine. This result has been shown to be due to an interfering substance in the milk oxidase preparation.

5. The guanase of rat liver is inhibited but not completely by the quinimine. Uricase from pig liver is not affected by the drug.

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# THE OXIDATION OF MESCALINE AND CERTAIN OTHER AMINES\*

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Mescaline, 3,4,5-trimethoxyphenylethylamine, has been shown by Slotta and Müller (1) to be excreted in the urine in the form of the corresponding acid when it is fed to various animals. It was therefore of interest to study the oxidation of mescaline *in vitro* and compare it with that of tyramine and other amines such as  $\beta$ -phenylethylamine,  $\beta$ -phenyl- $\beta$ -oxyethylamine, and isoamylamine which are oxidized by the tyramine oxidase. The results showed that the introduction of methoxy groups into the tyramine molecule definitely affected the properties of the oxidation. Mescaline is the active substance of a Mexican cactus (*Anhalonium lewinii*) and produces color visions and other effects when taken internally. The oxidation of mescaline which takes place in the liver is therefore in all probability a detoxicating mechanism.

## EXPERIMENTAL

An active tyramine oxidase is found in the liver and kidney of the rat, guinea pig, cat, dog, and rabbit as well as in other animals which were not used in this investigation. Under optimal conditions for the oxidation of tyramine, the oxidation of mescaline was investigated in the tissues of the various animals. The liver or kidneys were chopped with scissors, ground with sand in 0.05 M phosphate buffer at pH 7.8 (approximately 1.0 cc. of buffer per gm. of tissue), and pressed through muslin. Rabbit liver oxidizes mescaline rapidly, rat and guinea pig liver and rabbit kidney very slowly, and rat and guinea pig kidney and cat and dog

\* Part of the expense of this work was defrayed by a grant from the Duke University Research Council.



liver and kidney not at all. For the further study of the oxidation the rabbit liver was used. A standard preparation was made by dialyzing the preparation made as above against distilled water until the preparation alone had almost no oxygen uptake. The liver of an adult 2 kilo rabbit yielded about 150 cc. of the dialyzed suspension. 1.0 cc. of this preparation at pH 7.8 and 37° can oxidize 1.0 mg. of mescaline sulfate (Hoffmann-La Roche) to 3,4,5-trimethoxyphenylacetic acid in 2 hours with the uptake of 80 c.mm. of oxygen.

If the liver suspension is centrifuged, a brown precipitate comes down leaving a cloudy solution which contains most of the hemoglobin. Both fractions have about equal activity in oxidizing tyramine and mescaline. The precipitate can be washed free of hemoglobin with phosphate buffer of pH 6.7 and centrifuged and finally suspended in buffer of pH 7.8 and this procedure causes only a slight loss of activity compared with that of the original precipitate, which represents, of course, less than 50 per cent of the activity of the original suspension. Except for certain cases, therefore, the original dialyzed suspension was used.

As stated above, the rabbit kidney oxidizes mescaline slowly. If the kidney suspension is dialyzed and centrifuged, the solid contains a very active tyramine oxidase but mescaline is not oxidized. The liquid which is cloudy and contains the hemoglobin oxidized tyramine more slowly but oxidized mescaline at about the same rate as the original suspension. For the purposes of comparing the activity of the solid and the liquid after centrifuging, the solid was made up to a volume equal to the liquid by the addition of phosphate buffer at pH 7.8. To sum up these facts, it is possible to get preparations that will oxidize tyramine but not mescaline but so far no preparation has been obtained that will oxidize mescaline but not tyramine. Certain other amines,  $\beta$ -phenylethylamine,  $\beta$ -phenyl- $\beta$ -oxyethylamine, and isoamylamine, are oxidized by all the preparations that oxidize tyramine.

In order to determine whether it was possible to separate the mescaline oxidation from the tyramine in rabbit liver the following experiments were carried out. The relative rates of tyramine and mescaline oxidation were measured after dialyzing for varying lengths of time and compared with a suitably diluted undialyzed control. Both oxidation rates decreased slightly with time but

to the same extent. Liver suspension which was stirred into 20 volumes of water formed a precipitate which settled out and was washed with water several times. This preparation was active in oxidizing both tyramine and mescaline but was unable to oxidize succinate, amino acids, choline, and lactate. A liver preparation was allowed to autolyze at 37° for 48 hours with toluene as a preservative, and the rates of oxidation for both tyramine and mescaline decreased equally. Finally, heating the preparation at different temperatures for varying lengths of time caused an equal loss in oxidation rates of both substances.

It was possible that the inability of certain tissues to oxidize mescaline might be caused by the fact that the affinity constant of the enzyme for mescaline was less than that for tyramine, but this is not the case. Kohn (2) has measured the affinity constant for tyramine using a purified pig liver preparation. He obtained a half saturation value with a tyramine concentration of  $0.5 \times 10^{-3}$  M, and a saturation value of  $2.5 \times 10^{-3}$  M. We have been able to confirm this for the rabbit liver preparation and have been able to show that the saturation value for mescaline is  $0.25 \times 10^{-3}$  M; in other words, its affinity constant is greater than that for tyramine. No accurate half saturation values could be obtained.

Bernheim (3) showed that tyramine took up 1, 2, or 4 atoms of oxygen per molecule depending on the age and treatment of the tissue used. Kohn (2) was able to purify the enzyme so that only 1 atom was taken up. The rabbit preparation used in these experiments causes tyramine to take up 1 atom of oxygen very rapidly; the slope of the curve then abruptly changes and the uptake of the 2nd atom takes place more slowly and usually not more than 30 per cent of the 2nd atom is taken up during the experimental period. Mescaline, however, takes up 2 atoms and there is no change in the slope of the curve after the 1st atom of oxygen has been taken up. The oxidation of several other amines was therefore investigated. The results showed that the same preparation under the same conditions caused an uptake of 2 atoms of oxygen in the case of  $\beta$ -phenylethylamine and  $\beta$ -phenyl- $\beta$ -oxyethylamine with no change in slope. With isoamylamine it is necessary to have the correct enzyme-substrate relationship, which had to be determined for each preparation. If too little

enzyme was present in relation to the substrate, the oxidation ended abruptly before the theoretical uptake for 1 atom was reached. If too much enzyme was present, there was a tendency for somewhat more than 1 atom to be taken up. But in a small but definite range of concentration exactly 1 atom of oxygen was utilized for every molecule of isoamylamine. Fig. 1 shows the experimental oxygen uptakes in relation to the theoretical uptakes for all five amines and also shows their relative oxidation rates when saturation concentrations were used in all cases. The relative positions of the curves are not changed by using the amines in amounts which in each case will give an uptake of 100 c.mm. of oxygen. Hordenine, putrescine, and cadaverine are attacked slowly by the liver preparation but their oxidation rates are too slow for the determination of a definite end-point.

The fact that mescaline takes up 2 atoms of oxygen indicates that the corresponding acid is formed. In order to prove this 100 mg. of mescaline sulfate were oxidized by 50 cc. of the dialyzed rabbit liver preparation at pH 7.8. At the end of the oxidation a small amount of acetic acid was added until the proteins precipitated. The flask was then put in boiling water for 5 minutes and the coagulum filtered off. The filtrate was evaporated to about 10 cc. at room temperature under a fan. Enough hydrochloric acid was then added to make the solution about 0.2 N and any precipitate which formed was filtered off. The solution was then thoroughly extracted with ether. Occasionally when the ether evaporated off, crystals were left. Usually, however, an oil remained which was dissolved in the minimal amount of benzene. To this an excess of low boiling petroleum ether was added, the mixture was cooled, and the crystals filtered off. Recrystallization was carried out in the same way. Light yellow crystals were obtained which melted at 116°, uncorrected. The analysis was, theoretical, C 58.45, H 6.2; found, C 58.82, H 6.3. The yield was 56 per cent.

Hare (4) has shown that oxidation of tyramine is insensitive to cyanide during the uptake of 1 atom of oxygen per molecule but that the uptake of the 2nd atom was cyanide-sensitive (3). Mescaline takes up 2 atoms of oxygen per molecule, so cyanide was added to see whether the uptake of the 2nd atom was cyanide-sensitive. Surprisingly, cyanide completely inhibited the oxygen

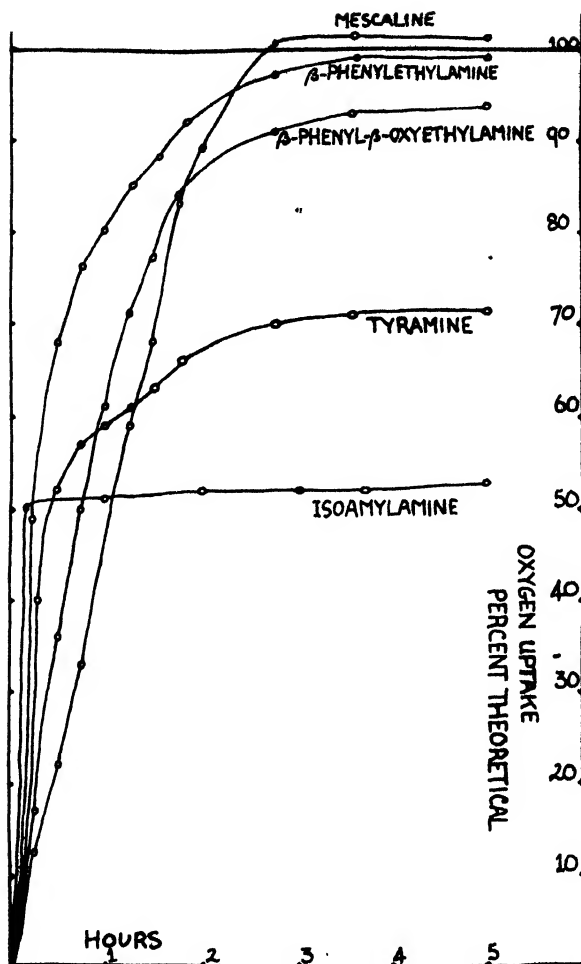


Fig. 1. The oxidation of the five amines by the rabbit liver preparation at pH 7.8. The oxygen uptakes are in per cent of the theoretical, assuming the utilization of 2 atoms of oxygen per molecule of amine. 1.0 mg. of each amine was used.

uptake in concentrations that had no effect on the uptake of the 1st atom of oxygen by tyramine in the same preparation. Thus 0.01 M KCN had no effect on the oxidation of tyramine,  $\beta$ -phenyl-

ethylamine,  $\beta$ -phenyl- $\beta$ -oxyethylamine, and isoamylamine but completely inhibited that of mescaline. In order to confirm this a cyanide concentration curve was plotted and the results are shown in Fig. 2. KOH was not used in the vessels, so that in each

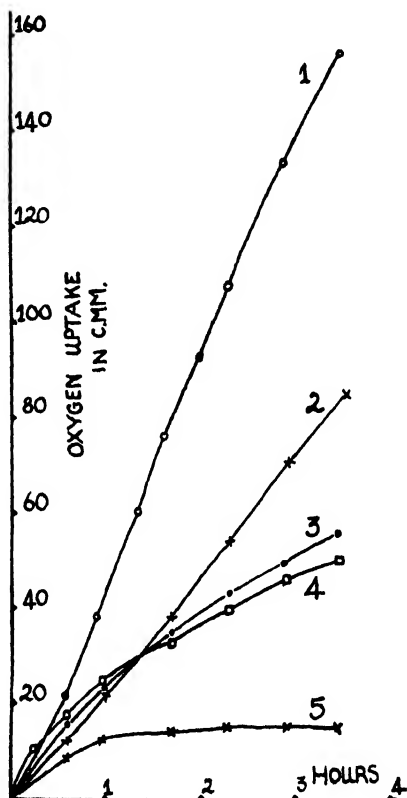


FIG. 2. The effect of various drugs on the oxidation of 2.0 mg. of mescaline sulfate by the rabbit liver preparation at pH 7.8. Curve 1 control, Curve 2 with 0.001 M potassium cyanide, Curve 3 with 0.008 M sodium borate, Curve 4 with 0.1 M potassium pyrophosphate, Curve 5 with 0.005 M potassium cyanide.

case the original cyanide concentration remained constant except for losses due to its oxidation. As the concentration was lowered, the inhibition became less until it was only about 40 per cent when 0.001 M KCN was used. A true cyanide-sensitive system

should be inhibited almost completely with a concentration of 0.001 M KCN. The indication here is that cyanide may be displacing the mescaline from the surface of the enzyme. That the enzyme belongs to the cyanide-insensitive group is indicated by the methemoglobin formation by  $H_2O_2$  when hemoglobin is added. Bernheim and Michel (5) have shown that methemoglobin is only formed under these conditions when cyanide-insensitive systems are used and mescaline produces as much methemoglobin as tyramine does. In a typical experiment when tyramine and mescaline had taken up the same amount of oxygen, 14.0 mg. of extra methemoglobin were formed by the tyramine and 13.5 mg. by the mescaline. The cyanide inhibition is obtained whether the cyanide is added to the enzyme before the mescaline or after it; in other words, the presence of the substrate does not protect the enzyme from the cyanide effect in the sense that xanthine protects xanthine oxidase from it (6). Fig. 2 shows that 0.1 M pyrophosphate and 0.008 M borate also inhibit the mescaline oxidation. In these concentrations the two drugs have no effect on the oxidation of tyramine or of the other three amines. The inhibitory effect of borate on mescaline makes it impossible to plot an accurate pH curve in the alkaline range. As in the case of tyramine, the uptake of the 2nd atom of oxygen by  $\beta$ -phenylethylamine and  $\beta$ -phenyl- $\beta$ -oxyethylamine is inhibited by 0.005 M KCN. With lower concentrations the inhibition is incomplete.

Kohn (2) made the interesting discovery that the rate of oxidation of tyramine was increased by increasing the oxygen tension. We have been able to confirm this and have tested the effect of increased oxygen tension on the rate of oxidation of the other amines. Table I shows the results. The oxidation rates of  $\beta$ -phenylethylamine and  $\beta$ -phenyl- $\beta$ -oxyethylamine and mescaline are increased by raising the oxygen tension from 20 per cent to 100 per cent. Hordenine, putrescine, and cadaverine are only slightly affected by increase in oxygen tension. Isoamylamine again shows a peculiarity. Increased oxygen tension increases the rate of oxidation but definitely depresses the amount of oxygen taken up, so that the oxidation stops sharply at less than 1 atom but not at any definite fraction of 1 atom. The explanation for this and also for the sensitivity of the oxidation to the correct enzyme-substrate relationship probably lies in the toxicity of the

end-product for the enzyme. Oxygen itself is not toxic to the enzyme, because it is possible to shake it in 100 per cent oxygen for at least 2 hours before adding the substrate without a decrease in its activity.

The ammonia produced by the deamination of the mescaline and the other amines was estimated by a vacuum distillation method and nesslerization after the oxidation was complete. The recoveries of ammonia in per cent of the theoretical were as follows: mescaline, 94 per cent;  $\beta$ -phenylethylamine, 97 per cent;

TABLE I

*Typical Experiment Showing Effect of Oxygen Tension on Rate of Oxidation of Various Amines by Rabbit Liver Preparation, pH 7.8*

Amine	O <sub>2</sub> uptake		Increase	Time
	20 per cent O <sub>2</sub>	100 per cent O <sub>2</sub>		
	<i>c.mm.</i>	<i>c.mm.</i>	<i>per cent</i>	<i>min.</i>
Tyramine	14	66	370	5
	56	137	145	15
Mescaline	4	14	250	5
	20	35	75	15
$\beta$ -Phenylethylamine	18	84	366	5
	54	187	264	15
$\beta$ -Phenyl- $\beta$ -oxyethylamine	6	28	366	5
	21	61	191	15
Isoamylamine	31	63	204	5
	89	113	27	15
	179	132	-26	35
	225	134	-40	60

$\beta$ -phenyl- $\beta$ -oxyethylamine, 92 per cent; isoamylamine, 108 per cent; tyramine, 72 per cent. The figures are the average of at least two determinations. All except tyramine show satisfactory agreement with the theoretical. The tyramine yields were consistently low, and the reason for this discrepancy has not been determined.

#### DISCUSSION

The introduction of methoxy groups into the benzene ring of tyramine definitely modifies the molecule in respect both to its

oxidative deamination by tissues and its pharmacological action. In comparison with tyramine the oxidation rate of mescaline is slow but the corresponding acid is formed by preparations which are able to form only small amounts of the acid from tyramine. The two phenylethylamines are also oxidized to their corresponding acids. These facts suggest that a free hydroxy group in the para position inhibits the oxidation of the aldehyde group formed after deamination, but has no effect, as the relative oxidation rates of tyramine and  $\beta$ -phenylethylamine show, on the deamination itself. The rate of deamination is, however, affected not only by methoxy groups in the ring but also by a hydroxy group in the  $\beta$  position as the relative oxidation rates of  $\beta$ -phenylethylamine and  $\beta$ -phenyl- $\beta$ -oxyethylamine show.

The fact that only the tyramine oxidase preparation of rabbit liver is able adequately to oxidize mescaline under the conditions of our experiments indicates that some factor other than the oxidase itself is necessary. This is also indicated by the fact that cyanide, pyrophosphate, and borate inhibit the mescaline oxidation in concentrations that leave the oxidation of tyramine and the other amines unaffected. Since all these substances form complexes with heavy metals, it is possible that some heavy metal is necessary with the tyramine oxidase to effect the oxidation of mescaline. This would be different from the cytochrome-indophenol oxidase system, for the latter is inactivated by prolonged dialysis and extensive washing as shown by the inability of the preparations to oxidize succinate. It is also thermolabile, because addition of boiled rabbit liver extracts to tyramine oxidase of other tissues does not enable them to oxidize mescaline.

It has been shown by Dakin (7) that methyl tyrosine is apparently metabolized differently from tyrosine itself. The oxidation of mescaline when compared with that of tyramine shows that methoxy groups in the benzene ring of amines closely related to tyrosine can affect the rate and course of the oxidation of these compounds *in vitro*.

#### SUMMARY

1. The oxidation of mescaline by a rabbit liver preparation has been described. This preparation contains an active tyramine oxidase but the tyramine oxidase in the tissues of several other animals is unable to oxidize mescaline.



2. Mescaline is oxidized to the corresponding acid which has been isolated. The theoretical amount of ammonia was recovered.

3. Unlike the oxidation of tyramine, the oxidation of mescaline is inhibited by pyrophosphate, borate, and relatively large concentrations of cyanide. Like that of tyramine the oxidation rate of mescaline is increased by raising the oxygen tension. Hydrogen peroxide is produced during the oxidation.

4. The properties of the oxidation of  $\beta$ -phenylethylamine,  $\beta$ -phenyl- $\beta$ -oxyethylamine, and isoamylamine are similar to those of tyramine.

*Addendum.*—Since this paper was submitted, Richter (8), Blaschko, Richter, and Schlossmann (9), and Pugh and Quastel (10) have published results on the oxidation of tyramine and other amines. Richter has isolated aldehydes after the oxidation of various amines. Blaschko *et al.* have studied the specificity and distribution of the enzyme and identified it with "adrenalin oxidase." See Blaschko, Richter, and Schlossmann for reference to Blaschko's earlier work. Pugh and Quastel have studied amine oxidation in the brain. Although these workers mention mescaline, they have not studied it in detail and consequently have not shown that the properties of its oxidation differ from those of the other amines.

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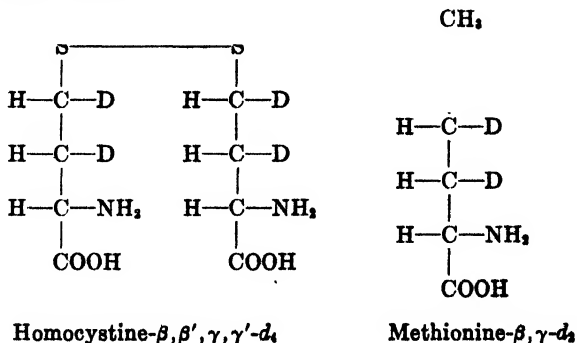
# THE SYNTHESIS OF TETRADEUTEROHOMOCYSTINE AND DIDEUTEROMETHIONINE

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The usefulness of deuterium as a means of labeling a compound in order to follow the substance through various steps in metabolism has been amply demonstrated by the researches of Schoenheimer and associates, particularly in the field of sterol and fat metabolism (1). The extension of the method to other fields of intermediary metabolism is, of course, obvious, and it occurred to us that such a method of approach might be useful in studying the metabolic relationship of methionine, homocystine, and cystine. In the particular study which we contemplated, homocystine and methionine were desired with deuterium attached to the  $\beta$  and  $\gamma$  carbon atoms.



In order to make this and allied studies possible, the synthesis of these deuterium derivatives of homocystine and methionine was therefore undertaken. In considering various possibilities for the synthesis of these compounds, we finally concluded that the best approach would be through dideuteroethylene bromide. We

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felt that, if the dideuteroethylene bromide could be prepared, the successful synthesis of the compounds desired was assured through the application of reactions we had already worked out for the synthesis of ordinary homocystine (2). To obtain the dideuteroethylene bromide we decided to prepare deuterioacetylene from calcium carbide and heavy water, and then to try to reduce the deuterioacetylene to dideuteroethylene, converting the latter, in the usual fashion, to the bromide.

The preparation of the deuterioacetylene was based on the method used by Zanetti and Sickman (3). The acetylene was collected over water, since the next step was to be an aqueous reaction, and there was no need of keeping the acetylene dry as was necessary in the work referred to. The method was furthermore adapted to the production of large quantities of the compound. The reduction of the deuterioacetylene was accomplished by chromous chloride in HCl solution, the reaction proceeding very smoothly. After the conversion of the deuterioethylene to the deuterioethylene bromide, it was found that an over-all yield of 63 per cent of the latter was obtained, based on the amount of deuterium oxide originally used. The dideuteroethylene bromide was condensed with benzyl mercaptan to yield dideutero-benzylthiolethyl bromide and this was then condensed with malonic ester. The dideutero-benzylthiolethylmalonic ester was saponified, and the resulting acid was brominated, after which the bromine was replaced with an amino group. The dideutero-benzylhomocysteine was next obtained in excellent yield by heating the dideutero-benzylthiolaminomalonic acid with HCl to split out CO<sub>2</sub>. The dideutero-benzylhomocysteine was then converted to the tetradeuterohomocystine by reduction of the former in liquid ammonia with metallic sodium followed by oxidation. The dideuteromethionine was obtained by reduction and methylation in liquid ammonia. Both the dideuteromethionine and the tetradeuterohomocystine so obtained contained the theoretical amounts of deuterium.<sup>1</sup>

The synthesis of these amino acids with the theoretical deuterium content is particularly significant to the question of the

<sup>1</sup> We wish to express our sincere appreciation to Dr. Rudolf Schoenheimer and to Dr. Mildred Cohn to whom we are deeply indebted for the deuterium analyses.

lability of deuterium, upon which attention has recently been focused (4). The many rather drastic reactions in aqueous solution and in other solvents containing ordinary hydrogen compounds are striking evidence of the stability of the deuterium in the particular positions we have dealt with. The theoretical quantity of deuterium in the compounds also assures us that the deuterium has remained in the desired positions, and that rearrangement or shifting of the deuterium has not taken place. If the deuterium had shifted to the nitrogen of the amino group or to the carboxyl, it would have been lost during the hydrolysis with HCl or during recrystallization from  $H_2O$ . That a deuterium atom has shifted to the carbon atom  $\alpha$  to the carboxyl group is also highly improbable. If such a shift had occurred, one would expect that some hydrogen would have partially replaced the deuterium.

It might also be pointed out that the very simple preparation of dideuteroethylene and dideuteroethylene bromide which we have reported here throws open a convenient approach to the synthesis of other amino acids and a variety of other organic compounds containing deuterium.

#### EXPERIMENTAL

*Preparation of Benzylthiolethylmalonic- $\beta,\gamma$ - $d_2$  Acid*—For the preparation of dideuteroacetylene 100 gm. of calcium carbide were evenly distributed in tubes *A* and *B* as shown in Fig. 1, and a 25 gm. tube of  $D_2O$  (99.65 per cent  $D_2O$ ) was opened and quickly attached at *W*. By varying the pressure of  $CO_2$  small amounts of  $D_2O$  could be readily introduced into tube *A*. The purpose of tube *B*, containing  $CaC_2$ , was to prevent the loss of any  $D_2O$  that might escape along with the deuteroacetylene from tube *A*. The deuteroacetylene which was generated was collected in bottle *D* which was filled with tap water. The water thus displaced from *D* was collected in bottle *E*.

Only a few liters of deuteroacetylene had collected in *D* by the time that all of the  $D_2O$  had been forced into tube *A*. The latter was then warmed gently at that portion of the tube containing the calcium deuterioxide and absorbed  $D_2O$ , and in this way the unchanged  $D_2O$  was slowly driven onto fresh  $CaC_2$ .

When approximately 2.5 liters of water remained in bottle *D*,

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the generation of deuterioacetylene was interrupted, the connection *Y* was closed with a screw clamp, and the 2.5 liters of water were removed to *E* from *D* by applying suction at *H*. Connection *Z* was then closed and *D* was replaced by bottle *E* and another bottle was then used in place of *E* to collect the water forced out of the collecting bottle by the further generation of deuterioacetylene. The loss of deuterioacetylene through its solubility in water was minimized by use of the same water in successive runs. The furnace was now heated gradually above

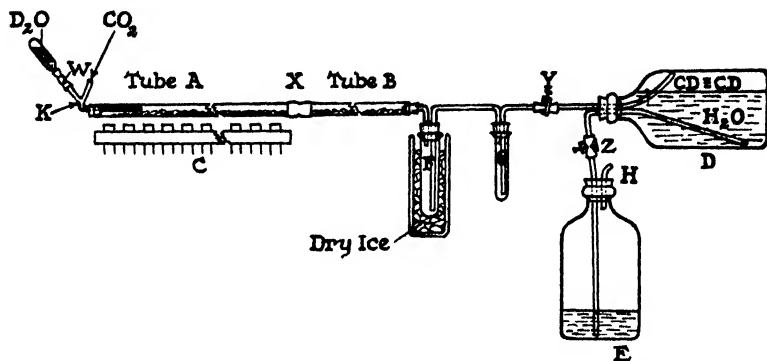


FIG. 1. Diagrammatic sketch of deuterioacetylene generator. *A* and *B* represent combustion tubes, Pyrex No. 172, 900 × 19 mm. The tubes are connected with heavy walled rubber tubing at *X*; *C* is a gas furnace with which any desired portion of *A* can be heated; *D* and *E*, 5 gallon Pyrex bottles; *F* and *G*, large Pyrex test-tubes. *F* is immersed in a dry ice bath to prevent any water vapor from diffusing back into *B* from *D*; *H*, air outlet; *K*, *Y*-tube. The lower branch extends through a rubber stopper into *A* for a distance of 6 inches, and is constricted about 1 inch from the outlet; *W*, *Y*, *Z*, heavy walled rubber tubing.

the decomposition point of calcium deuterioxide, in such a way that all the liberated deuterium oxide was driven onto fresh  $\text{CaC}_2$  in tube *B*. Meanwhile a very slow stream of  $\text{CO}_2$  was allowed to enter the system at *K*. When all the deuterium oxide had been removed from tube *A*, tube *B* was then placed in the furnace and the gradual process of converting the calcium deuterioxide back into  $\text{D}_2\text{O}$ , etc., continued until there was an insignificant amount of deuterium left in *B* in the form of calcium deuterioxide. The combustion tube was finally swept out with  $\text{CO}_2$  in order to get all of the deuterioacetylene into the collecting bottle.

The dideuteroacetylene was reduced to dideuteroethylene with chromous chloride (5). The solution of chromous chloride was prepared by reducing 2 pounds of chromic chloride ( $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ ) in 1800 cc. of 25 per cent HCl with 1 pound of mossy zinc.

The chromous chloride solution was added to bottle *D* by allowing the partial vacuum which had previously been created in the bottle to draw in the solution. The bottle containing the deuterioacetylene and the chromous chloride solution was shaken vigorously for 6 hours. The second bottle was also treated in this manner.

In order to convert the dideuteroethylene into dideuteroethylene bromide, the reduced gas was absorbed in three gas-washing towers containing an excess of bromine. The towers were kept in an ice bath during the process. The dideuteroethylene bromide, containing some bromine, was washed with an ice-cold solution of sodium bisulfite until colorless. After being dried over anhydrous sodium sulfate, the resulting product was distilled. The material boiling within the range  $130\text{--}135^\circ$  was used for the synthesis of  $\beta$ -benzylthioethyl- $\alpha, \beta$ - $d_2$  bromide. From the 25 gm. of  $\text{D}_2\text{O}$ , 150 gm. of dideuteroethylene bromide were obtained which is 63 per cent of the theoretical yield.

25 gm. of benzyl mercaptan were added to 5 gm. of sodium in 40 cc. of methyl alcohol. When the sodium had all dissolved, the solution was cooled in an ice bath. At the same time, 199 gm. of dideuteroethylene bromide were cooled to its freezing point in a 1 liter flask. The two solutions were mixed and kept in an ice bath. In less than 1 minute a vigorous reaction occurred. Immediately, 300 cc. of water were added. The lower layer was distilled at 70 mm. until all the unchanged deuterioethylene bromide had been removed. This amounted to 152 gm. The condensation just described was repeated with the recovered deuterioethylene bromide, 5 gm. of sodium and 25 gm. of benzyl mercaptan being used. 113 gm. of unchanged dibromide were recovered from this second run and used in a third condensation with 4.1 gm. of sodium and 22 gm. of benzyl mercaptan. 78 gm. of dideuteroethylene bromide were recovered from this last run. The residues left from these vacuum distillations of all three condensations were then combined and distilled at 1 mm. pressure. The fraction boiling from  $115\text{--}130^\circ$ , consisting chiefly of  $\beta$ -benzylthioethyl- $\alpha, \beta$ - $d_2$  bromide, was redistilled. The frac-

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tion boiling at 118–123° at 1 mm. pressure, with a bath temperature of 150°, weighed 55 gm., a yield of 41 per cent of the theoretical amount.

To a solution of 5.5 gm. of sodium in 100 cc. of absolute alcohol were added 60 gm. of malonic ester. The solution was cooled until precipitation began and then 55 gm. of dideuterobenzylthiolethyl bromide were introduced. The mixture was allowed to stand at room temperature for an hour, and then refluxed for 3 hours. The reaction mixture was hydrolyzed by refluxing for 1 hour with 70 gm. of KOH in 300 cc. of 50 per cent alcohol. The alcohol was removed *in vacuo* and the residual solution acidified with concentrated HCl below 20° in the presence of 200 cc. of alcohol-free ether. 200 cc. of benzene were added to the ether layer and the ether removed *in vacuo* until crystallization commenced. After cooling the mixture in an ice bath for a short time the product was filtered, washed with benzene, and air-dried. 28.5 gm. of the compound were obtained. It melted at 119–120° (corrected). Concentration of the mother liquors yielded 11 gm. more of the product which melted a few degrees lower. The total yield was 39.5 gm., which is 65 per cent of the theoretical amount. The compound possessed the following analysis.

$C_{12}D_2H_{12}O_4S$ . Calculated, D 14.3 atom %; found, D 14.3  $\pm$  0.4 atom %

*Preparation of S-Benzylhomocystine- $\beta, \gamma$ - $d_2$* —28.5 gm. of  $\beta$ -benzylthiolethyl- $\alpha, \beta$ - $d_2$ -malonic acid were brominated and aminated as described for S-benzylhomocystine (2). A yield of 16.8 gm. was obtained. This is 66 per cent of the theoretical amount. The product is indistinguishable from the ordinary S-benzylhomocystine in melting point and crystalline appearance. The compound gave the following analytical values.

$C_{11}D_2H_{12}O_2NS$	Calculated.	N 6.16, S 14.11, D 13.3 atom %
	Found.	" 6.29, " 14.00, " 13.4 $\pm$ 0.5 atom %

*Preparation of Homocystine- $\beta, \beta', \gamma, \gamma'$ - $d_4$* —8.25 gm. of S-benzylhomocystine- $\beta, \gamma$ - $d_2$  were reduced with sodium in liquid ammonia as previously described for ordinary S-benzylhomocystine (6). After the reduction was complete, 3.5 gm. of  $NH_4Cl$  were added during the evaporation of the  $NH_3$ . The residue was dissolved in  $H_2O$  and oxidized with air in the presence of ferric ion as a

catalyst. The crude product was dissolved in the minimum amount of dilute NaOH, filtered, and precipitated with HCl. The yield was 4.4 gm., which is 90 per cent of the theoretical amount. The compound gave the following analysis.

$C_2D_4H_{12}O_2N_2S_2$ .	Calculated.	N 10.29, S 23.55, D 25.0 atom %
	Found.	" 10.62, " 23.48, " $24.4 \pm 0.3$ atom %

*Preparation of Methionine- $\beta$ , $\gamma$ - $d_2$* —8.5 gm. of S-benzylhomocysteine- $\beta$ , $\gamma$ - $d_2$  were reduced with sodium in liquid ammonia until the solution had a permanent blue color which was discharged with 0.3 cc. of  $CH_3I$ . Then a further quantity of 2.4 cc. (1.05 moles) of  $CH_3I$  was added. The residue left after the removal of the  $NH_3$  was dissolved in 15 cc. of  $H_2O$  and made just alkaline to litmus with 45 per cent HI. 4.5 gm. of halogen-free product precipitated. The filtrate was concentrated almost to dryness and diluted to 200 cc. with absolute alcohol. 0.9 gm. of material precipitated on standing in the ice box overnight. Recrystallization of the two fractions from water by the addition of alcohol gave 4.4 gm. of halogen-free methionine- $\beta$ , $\gamma$ - $d_2$ . The total yield of pure material was 78 per cent of the theoretical amount. The following analytical values were obtained for the compound.

$C_2D_2H_8O_2NS$ .	Calculated.	N 9.27, S 21.21, D 18.2 atom %
	Found.	" 9.42, " 21.05, " $17.9 \pm 0.2$ atom %

#### SUMMARY

The synthesis of tetradeuterohomocystine and of dideuteromethionine has been described.  $CaC_2$  was allowed to react with deuterium oxide to form dideuteroacetylene which was then reduced by chromous chloride to dideuteroethylene and this in turn converted to the dibromide. The dideuteroethylene bromide so formed was condensed with benzyl mercaptan to yield the dideutero benzylthioethyl bromide. By means of the malonic ester amino acid synthesis, the latter compound was converted to dideutero benzylhomocysteine. This compound served for both the synthesis of tetradeuterohomocystine and dideuteromethionine. The former was prepared by reduction in liquid ammonia with sodium followed by oxidation, and the latter by means of reduction and subsequent methylation. Both compounds contained the theoretical content of deuterium.



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The possible utilization of the dideuteroethylene and its bromide in the synthesis of other amino acids and other organic compounds containing deuterium in definite positions has been pointed out.

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## THE SALT EFFECT ON THE HEMOGLOBIN-OXYGEN EQUILIBRIUM

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The equilibrium between hemoglobin and oxygen seemed to have been satisfactorily analyzed by the work of Henderson (1) and Peters and Van Slyke (2). Under the influence of Henderson's theory, that this equilibrium depends on the difference in the strength of the acid dissociation constants of hemoglobin and oxyhemoglobin, the specific effect of the added salts was ignored and any inhibition was usually attributed to the hydrogen ion concentration. As a consequence many investigators of this problem were often overzealous in having their solutions well buffered. The discovery of Henriques (3) of the probable existence of "carbhemoglobin" was followed by a number of investigations demonstrating the existence of carbamino compounds of hemoglobin and oxyhemoglobin. The carbamate equilibrium was thus given an important function in CO<sub>2</sub> transport (Ferguson and Roughton (4), Stadie and O'Brien (5)). But up to the present time the effect of electrolytes has received scanty attention. Barcroft and Camis (6) and Barcroft and Roberts (7), as early as 1909, reported that sodium chloride and potassium chloride increased the affinity of hemoglobin for oxygen. More recently Green and Talbot (8) in a preliminary note stated that, "the oxygen dissociation curves of horse hemoglobin moved symmetrically to the right with increasing sodium chloride concentrations," and Kono (9) has reported that in low hemoglobin concentrations neutral salts have no influence on the oxygen dissociation, while in high hemoglobin concentrations these salts depress the dissocia-

tion equally when isotonic solutions are used. Clearly it is essential for a comprehensive treatment of the hemoglobin-oxygen equilibrium to restudy the problem of the effect of electrolytes. Such a study is presented in this paper which, it is believed, supports the opinion that hemoglobin combines with some anions, effecting the hemoglobin-oxygen equilibrium.

#### EXPERIMENTAL

*Preparation of Hemoglobin Solutions*—Defibrinated human blood was centrifuged four times and washed with 0.9 per cent sodium chloride solution. To the remaining cell suspensions 1.5 volumes of water and 0.1 volume of diethyl ether were added. The mixture was thoroughly shaken and then centrifuged again for 30 minutes. The thick mass containing the cell debris was discarded. To the remaining solution 0.2 volume of a colloidal suspension of aluminum hydroxide was added. This mixture was shaken, carefully filtered, and the excess ether removed by blowing a stream of oxygen over the surface of the solution contained in a bottle which was slowly rotated. The resulting solution of hemoglobin was placed in a cellophane bag (porous enough to let hemin diffuse out slowly) which was then introduced into a cylinder in which triple distilled water was kept flowing continuously for 36 hours. Usually, 20 liters of water were used in this process. In the final preparation for the spectroscopic determination the dialyzed human hemoglobin was centrifuged once more. No potassium, sodium, or chloride ion could be detected in these samples. Each step in the procedure was carried out in a cold room maintained at 3°, and solutions were stored at a temperature of 3–5°. Each sample was saturated with oxygen in a tonometer, and the concentrations of oxyhemoglobin present in these solutions were determined on 2 cc. samples in triplicate by the Van Slyke manometric method.

After some experience with these solutions we established the criterion that a well dialyzed solution should be saturated to the extent of 50 per cent at 25° with an oxygen pressure of 1 mm. Many samples which did not fulfil this condition were rejected. In no case was a sample obtained which was saturated to a greater extent, under these specified conditions.

*Preparation of Gas Mixtures*—Gas mixtures of known composi-



to pass over copper pellets maintained at 450–500° within the Pyrex tube in the furnace (*B*). The 3-way stop-cock (*C*) connected through one branch with the drying tubes (*D*), which were filled with soda lime and phosphorus pentoxide; the other branch (*C*) functioned as a convenient by-pass for water vapor during reactivation of the copper surface, or for purified undried nitrogen. The dried nitrogen was allowed to escape into the air through one branch of the 3-way stop-cock (*E*), or could be admitted to the gas-mixing chamber (*F*).

Commercial medicinal oxygen was also passed over soda lime and phosphorus pentoxide through stop-cock *I*. The oxygen-drying tubes (*G*) were connected to the mixing chamber (*F*) in the same manner, through *H*, as for nitrogen, and stop-cock *I* made undried oxygen available.

Commercial carbon dioxide was freed of oxygen and other contaminants by adsorption upon activated charcoal contained in a trap (*J*) cooled with acetone and solid carbon dioxide. Prior to adsorption of the carbon dioxide the adsorption trap and phosphorus pentoxide drying chamber (*K*) were evacuated through stop-cock *N'* by the pumps. The charcoal was degassed by heating with a furnace to 500° in a high vacuum. The evolution of the carbon dioxide from the charcoal surface was controlled by lowering the cooling bath around trap *J*. The dried gas was admitted to the mixing chamber through the 3-way stop-cock *L*.

The gas-mixing chamber (*F*) consisted of one 6 liter and two 12 liter flasks connected, but made independent of each other by means of stop-cocks *M* and *M'*. One (or more) of these flasks could be evacuated through stop-cock *N*, to a pressure of  $10^{-5}$  mm. of mercury. The pressure of oxygen in the gas mixtures used was determined directly by the use of the McLeod gage (*O*) calibrated to measure pressures accurately over the range of  $10^{-3}$  mm. to 20 mm. of Hg. Higher pressures were read directly from the closed end manometer at *P*.

The final mixture was made by first admitting the oxygen (and carbon dioxide) to a definite pressure, and then adding the diluent gas until the pressure in the chamber was atmospheric. The mixture was then allowed to reach equilibrium temperature and the final pressure was recorded. Portions of the equilibrium mixture of gas were then passed through the Toepler pumps (*Q*) and

thereby forced through the mercury valve (*R*) into the tonometer absorption cell.

The nitrogen and carbon dioxide obtained by treatment in this apparatus were tested for the absence of oxygen by passing the gases through a solution of safranine which had been reduced by hydrogen in the presence of platinized asbestos (10).

*Tonometer Absorption Cell and Tonometer pH Cell*—In the study of the hemoglobin-oxygen equilibrium by the spectrophotometric method it is necessary to bring the hemoglobin solution into equilibrium with a known pressure of oxygen in the same cell to be used for measuring the absorption spectrum. The tonometer cell used for this purpose is shown in Fig. 2. The saturation chamber (*E*) was filled to a point just below the ground joint

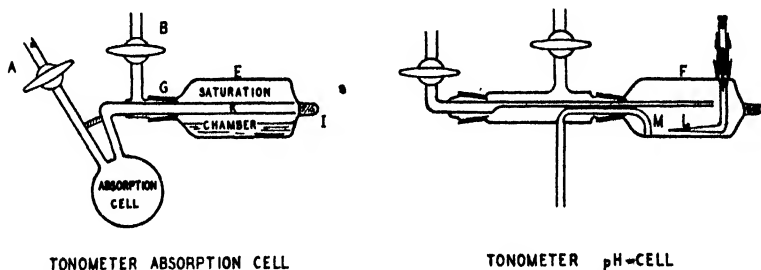


FIG. 2. Absorption and pH tonometers

(*G*) with the solutions to be measured. The tonometer absorption cell fitted into a socket on a base upon which a motor with a 144 to 1 speed reduction was mounted. The shaft of the motor was coupled to tip *I* on the saturation chamber by means of a piece of rubber tubing. This arrangement allowed *E* to turn upon joint *G* at about 40 R.P.M. The gas mixture passed into the cell through stop-cock *B* and out at *A* while *E* was rotating. After the solution was saturated, the tonometer absorption cell was tilted so that the saturation chamber (*E*) was in a vertical position, and the incoming gas mixture forced the saturated solution up the tube (*K*) into the absorption cell, where its optical absorption could be measured. The solution could be transferred back into the saturation chamber by changing the inlet tube from *B* to *A* and tilting the cell so that the liquid ran back through *K*. Stop-cocks *A* and *B* had plugs bored in such a way that the tubing

up to the stop-cock could be swept out with the gas mixture, thus preventing contamination of the contents of the cell with the air originally present.

The tonometer pH cell consisted of a saturation chamber (*F*) which contained a capillary glass electrode (*L*) and an agar-potassium chloride bridge (*M*). It was manipulated in a manner similar to that for the tonometer absorption cell. The saturating gas mixture was passed, first through the tonometer absorption cell, and then through the tonometer pH cell as both saturation chambers were being rotated. While the optical absorption was being measured, the pH was measured in the other cell. The electrical measurements involving the glass electrode were made in the usual manner with an amplifier circuit which contained an FP-54 pliotron.

A Freas air thermostat contained the speed-reducing motor, the tonometer cells, a calomel electrode, and a gas-washing tube which could be filled with water or a suitable solution to prevent loss of water from the tonometer cells on passage of the gases. This thermostat controlled the temperature of the air within  $\pm 0.5^\circ$  at  $25^\circ$ . The gases employed were led into the thermostat by means of seamless copper tubing sealed to glass at one end by wax joints. Short lengths of seasoned rubber tubing served to connect the tonometer cell stop-cocks with the copper tube.

*Absorption Measurements*—The photoelectric spectrophotometer and the general method employed here to determine the spectroscopic constants and the relative amounts of hemoglobin and oxyhemoglobin in solution have been described in an earlier paper (11). Absorption cells with crystal quartz windows were used in a light-tight cell chamber fitted with an electrically driven fan, heating coil, water-cooled coils, and a mercury-filled thermostat which interrupted the current through a relay controlling the heating current. The absorption cells were thus held at constant temperature; in this case,  $25^\circ$ .

The absorption coefficients, as calculated from Beer's law, are defined by the equation:

$$\log_{10} \frac{I_0}{I_s} = \alpha cl$$

where  $I_0$  = relative intensity of light of wave-length  $\lambda$  passing through solvent

- $I_0$  = relative intensity of light of wave-length  $\lambda$  passing through solution to be studied  
 $\alpha$  = absorption coefficient  
 $c$  = concentration in moles per liter (based on total oxygen capacity)  
 $l$  = length of absorbing medium; i.e., length of absorption cell in cm.

The concentration employed in the calculation of the relative absorption coefficients was that of the total oxygen capacity in moles of oxygen per liter of the solution studied; i.e., the oxygen capacity of the initial solution as determined by the Van Slyke manometric method, multiplied by the appropriate dilution factor. Thus the absolute values of the absorption coefficients may be calculated from the figures given, only when the number of oxygen molecules per molecule of oxyhemoglobin is definitely known (very probably 4).

#### *Absorption Spectrum of Human Dialyzed Hemoglobin*

Numerous reports have been published on the absorption spectrum of hemoglobin (copious information on the literature will be found in Drabkin and Austin's paper (12)). Few of them (Newcomer (13), Suhrmann and Kollath (14)) examined it at the same time in the ultraviolet and visible regions of the spectrum. Adams (15), who made the most recent report on the ultraviolet spectrum of hemoglobin, stated that no difference was found between hemoglobin and oxyhemoglobin with respect to the position of the Soret band (around 4000 Å.), although, according to him, "The exact centre of the band is difficult to locate."

Dialyzed human hemoglobin solutions prepared as described above were examined in the ultraviolet and visible regions of the spectrum. The absorption spectrum was determined with cells open to the air, or in the tonometer absorption cell with an atmosphere of oxygen. Solutions of purified but undialyzed oxyhemoglobin in acetate, bicarbonate, phosphate, and veronal buffers, respectively, were studied in the same manner. No differences in the spectra so determined were found. For the absorption spectrum of hemoglobin, the solutions were deoxygenated with purified nitrogen, hydrogen, or carbon dioxide. Dialyzed hemoglobin and hemoglobin in the presence of the buffers mentioned above gave identical spectra (Fig. 3). In Table I is given the position



of the maximum absorption bands of oxyhemoglobin and hemoglobin. In both substances the positions of the first band, attributed to the globin fraction, are identical; the second band (3500 Å.), which has a sharp maximum in oxyhemoglobin, is

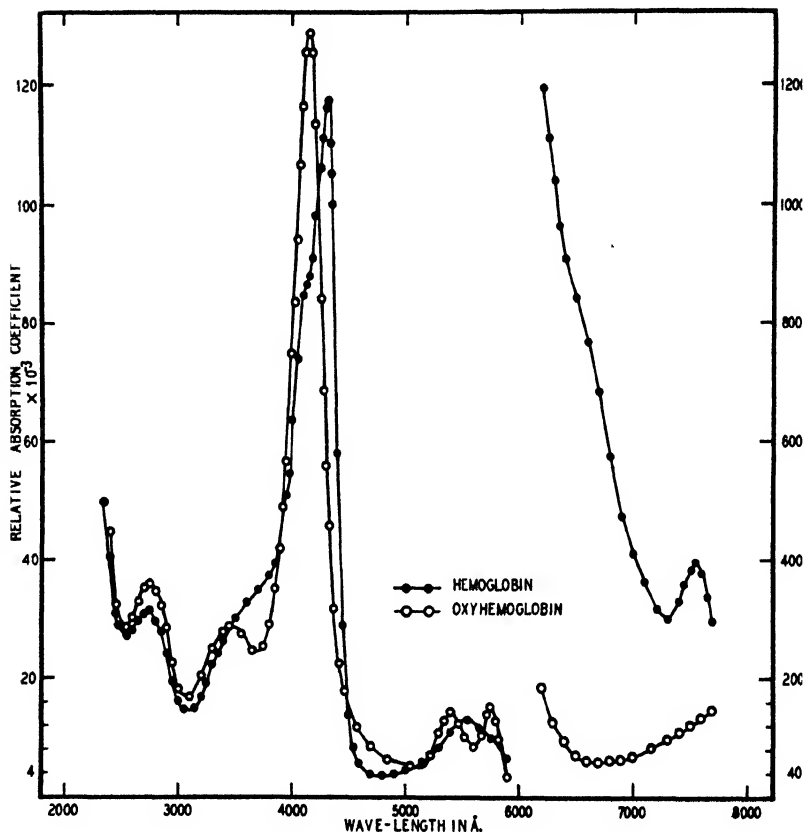


FIG. 3. Absorption spectra of hemoglobin and oxyhemoglobin

shifted to the right on deoxygenation, thus becoming masked by the Soret band; the Soret band shifts to the right by 150 Å. on deoxygenation. Finally, a band at 7550 Å. was observed in oxyhemoglobin, not reported previously probably because all the measurements heretofore had been stopped at 7000 Å.

*Analytical Region of Spectrum*—In all previous measurements of the hemoglobin-oxyhemoglobin equilibrium performed spectrophotometrically the fourth and fifth bands (5415 and 5760 Å. respectively) of oxyhemoglobin were used for the determination of the ratios of absorption coefficients. Both the absolute magnitudes and the ratio of the absorption coefficients at 7550 Å. make this the most useful wave-length in comparing the spectroscopic and the Van Slyke methods of analysis. In practise, the values of  $\log I_0/I_x$  for a solution of oxyhemoglobin, for the same

TABLE I  
Absorption Data for Hemoglobin and Oxyhemoglobin

Hemoglobin		Oxyhemoglobin	
Wave-length, Å.	Absorption coefficient	Wave-length, Å.	Absorption coefficient
Maxima			
2750	31,400	2750	36,000
4300	118,000	3500	28,800
5525	12,900	4150	128,500
7550	396	5415	14,200
		5760	15,400
Minima			
		2550	28,000
2550	27,000	3050	16,700
3100	14,400	3700	24,200
4750	3,680	5100	5,100
7300	290	5600	8,400
		6700	60

solution completely deoxygenated in the tonometer cell with nitrogen, and for the solution after equilibration with a gas mixture of known oxygen pressure were determined at 7550 Å. Calculation of the per cent oxygenation from the absorption data could be performed readily, either graphically or from the equation

$$\% \text{ oxygenation} = 100 \times \frac{\log \frac{I_0}{(I_x)_{\text{Hb}}} - \log \frac{I_0}{(I_x)_{\text{mixture}}}}{\log \frac{I_0}{(I_x)_{\text{Hb}}} - \log \frac{I_0}{(I_x)_{\text{HbO}_2}}}$$

*Effect of Salts on Hemoglobin-Oxyhemoglobin Equilibrium*—The experimental results on the effect of salts on the hemoglobin-oxyhemoglobin-oxygen equilibrium are found in Table II and Fig. 4. All determinations were made at 25°, and as nearly as possible the pH of the solutions was kept constant. The pH change of hemoglobin solutions on addition of neutral salts reported by Cohn, Green, and Blanchard (16) and observed by us

TABLE II  
*Hemoglobin-Oxygen Salt Effect Data*

$p(\text{O}_2) = 5 \text{ mm.}$

Salt	Concentration <i>M</i>	$\nu \times 100$	pH	$p\text{CO}_2$
$\text{NaHCO}_3$	0.0182	16.5	6.8	100
	0.0127	24.0		70
	0.00727	42.5		40
	0.00182	62.0		10
	0.000182	75.5		1
	0.00	80.5		0
$\text{NaCl}$	0.02	73.6	7.12	
	0.06	66.5	6.99	
	0.10	58.4	7.04	
	0.00	81.0	6.99	
$\text{Na}_2\text{SO}_4$	0.02	60.2	7.08	
	0.06	37.4	7.03	
	0.10	30.6	7.06	
$\text{Na}_2\text{C}_2\text{H}_3\text{O}_7$	0.02	44.5	7.15	
$\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$	0.005	66	6.8	
	0.02	36		
	0.05	20		
$\text{KCl}$	0.05	71	6.8	
	0.10	58.5		
	0.15	47.5		

was very small with the salt concentration used in these experiments; furthermore, the minimum of this pH change is around the isoelectric point of hemoglobin, pH value of our solutions. The hemoglobin concentration and the oxygen pressure (5 mm.) were kept constant, the nature of the salt and its concentration being the only variable factors. A glance at Fig. 4 will show that all salts inhibit, in varying degrees, the affinity of hemoglobin for oxygen; the most marked effect is that due to the bicarbonate.

These results at constant pH and constant oxygen pressure can be expressed by the empirical equation

$$\frac{K}{1 + K'(Sa^-)} \quad (1)$$

in which  $y$  is the fraction oxygenated and  $(Sa^-)$  is the concentration of the added salt. The curves in Fig. 4 are made on the basis of this equation, with  $K'$  values determined from the data in

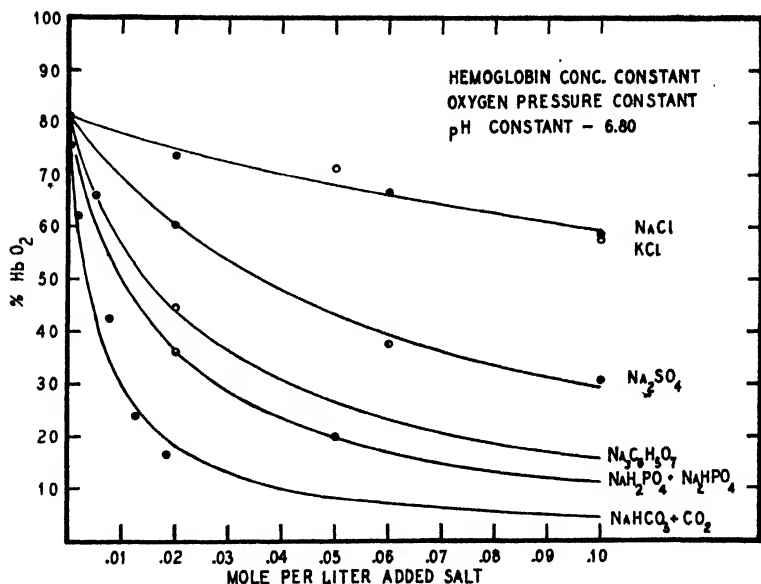


FIG. 4. Saturation of hemoglobin as a function of the salt concentration

Table II and with  $K$  taken to be equal to 0.8 (the value of  $y$  when no salt is added).

#### DISCUSSION

The possibility that the effect of salts on this equilibrium is due to the known effect of salts in changing the activity coefficients of the substances involved may be examined in the light of the work of Cohn and his coworkers, on the activity coefficients of the proteins, and particularly of Green (17) on the activity coefficients of carboxyhemoglobin.

If we assume that 4 oxygen molecules combine stepwise with hemoglobin, and if we take activity coefficients into account, the fractional amount of hemoglobin oxygenated ( $y$ ) may be expressed by the equation

$$y = \frac{K_1 \frac{\gamma_0}{\gamma_1} p + 2K_1 K_2 \frac{\gamma_0}{\gamma_2} p^2 + 3K_1 K_2 K_3 \frac{\gamma_0}{\gamma_3} p^3 + 4K_1 K_2 K_3 K_4 \frac{\gamma_0}{\gamma_4} p^4}{4 \left[ 1 + K_1 \frac{\gamma_0}{\gamma_1} p + K_1 K_2 \frac{\gamma_0}{\gamma_2} p^2 + K_1 K_2 K_3 \frac{\gamma_0}{\gamma_3} p^3 + K_1 K_2 K_3 K_4 \frac{\gamma_0}{\gamma_4} p^4 \right]} \quad (2)$$

where  $K_1$ ,  $K_2$ ,  $K_3$ , and  $K_4$  refer to the association constants for each of the steps of oxygen combination,  $p$ , the pressure of the oxygen, and  $\gamma_0$ ,  $\gamma_1$ ,  $\gamma_2$ ,  $\gamma_3$ , and  $\gamma_4$  are the activity coefficients of unoxygenated hemoglobin and each of the oxygenated species respectively. The depressing effect of salts would then be a function of  $\gamma_0/\gamma_x$  ( $\gamma_x$  referring to any of the oxygenated species) and  $\gamma_0/\gamma_x$  should have a value less than 1.

Green (17) from solubility studies found that the activity coefficient for carboxyhemoglobin could be expressed by the equation

$$-\log \gamma = K_i \sqrt{\mu} - K_o \mu \quad (3)$$

in which  $\mu$  is the ionic strength of the solution, and  $K_i$  and  $K_o$  are the salting-in and salting-out constants, respectively. " $K_i$  is characteristic of the protein and  $K_o$  varies with the electrolyte." From Equation 3 it follows that

$$-\log \frac{\gamma_0}{\gamma_x} = \Delta K_i \sqrt{\mu} - \Delta K_o \mu \quad (4)$$

If  $K_o$  were only a function of the salt as Green postulates, the last term in Equation 4 would be equal to 0 and it might be argued that  $\gamma_0/\gamma_x$  should have a value very nearly equal to 1, and that the salt effect is due rather to compound formation between the hemoglobin and the anion. However, the application of Equation 3 (developed for strong electrolytes) to proteins is very doubtful and any conclusion regarding compound formation must be made with some reserve.

By analogy to the activity coefficients of other proteins as determined by solubility measurements we may represent the activity coefficients of hemoglobin and oxyhemoglobin graphically (Fig. 5) as a function of the ionic strength. We are dealing with

the difference between these two functions in any case, whether there be compound formation or not.

To represent our results as a function of the ionic strength we shall simplify the problem and consider the hemoglobin equilib-

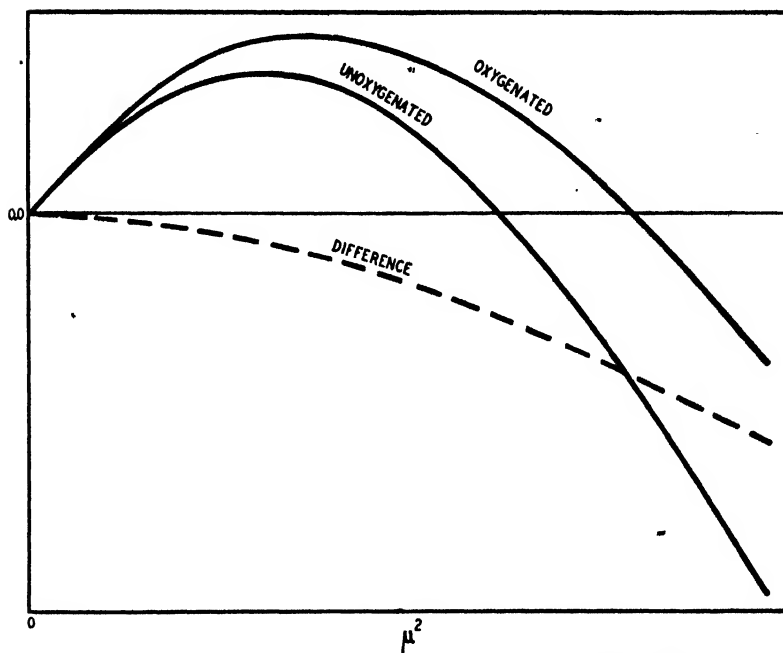


FIG. 5. Qualitative relationship between activity coefficients of hemoglobin and the ionic strength of the solution.

rium (at constant oxygen pressure and constant pH) to be represented by the equation



The equilibrium constant for this reaction is then

$$K = \frac{\gamma'(\text{Hb}(\text{O}_2)_n)}{\gamma_0(\text{Hb})} \quad (6)$$

$$-\text{p}K = \log \frac{\gamma'}{\gamma_0} + \log \frac{(\text{Hb}(\text{O}_2)_n)}{(\text{Hb})} \quad (7)$$

$\log \gamma'/\gamma_0$  is a function of the ionic strength and  $(\text{Hb}(\text{O}_2)_n)/(\text{Hb}) = y/(1 - y)$ . Plotting  $\log y/(1 - y)$  against  $\mu$ , the ionic strength, we obtain curves given in Fig. 6.

While the points for different salts fall on different curves, it cannot be argued conclusively that the effect is due to specific

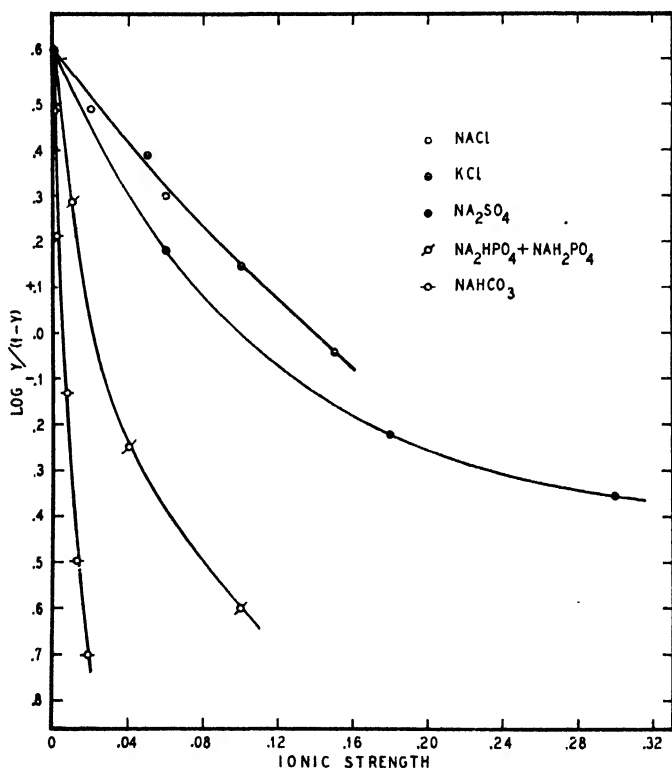


FIG. 6. The logarithm of the ratio of oxyhemoglobin to hemoglobin as a function of ionic strength.

compound formation until we know more about the effect of strong electrolytes on the activity coefficients of such complicated zwitter ions as the proteins. It is known that carbon dioxide depresses the affinity of hemoglobin for oxygen through the formation of carbamino compounds of hemoglobin and oxyhemoglobin, but hemoglobin at isoelectric pH does not form carbamate (Stadie and O'Brien (5)). In our experiments with bicar-

bonate, where the pH of the solution was kept at 6.8 (isoelectric point of hemoglobin pH 6.78 (18, 19)), this depressing effect of the bicarbonate-carbon dioxide mixture cannot therefore be attributed to carbamate formation. Nevertheless, we might reasonably suppose that no theory of interaction between negative ions and hemoglobin will account for the great difference between the bicarbonate and chloride ions, and therefore compound formation takes place between the bicarbonate and the hemoglobin. The same argument might also apply to the phosphate.

It is impossible to isolate the effect of the monohydrogen phosphate and dihydrogen phosphate ions without changing the pH of the solution, for at constant pH the activities of these two ions remain in a constant ratio to each other. The same condition holds for the bicarbonate ion, carbonate ion, carbonic acid, and carbon dioxide. The effects of the individual species can only be analyzed after we have a knowledge of the isolated effect of the hydrogen ion on the hemoglobin-oxygen equilibrium in dialyzed solution.

The empirical Equation 1 can be deduced on the assumption that one negative ion combines with unoxygenated hemoglobin to decrease its tendency to combine with oxygen to such an extent that only an inappreciable amount of oxygen combines with the salt form. However, in view of our concept of proteins as zwitter ions, such an assumption does not seem to be a reasonable one.

Throughout the whole of this work we have assumed that the absorption spectrum of one oxygenated heme is not dependent on the oxygenation of another heme on the same molecule; that the interaction energy between the heme groups on the hemoglobin molecule is not very large. We have found no evidence contradicting this assumption. In no case did we find an absorption region which failed to increase or decrease proportionately with increasing oxygenation, as would be expected if this assumption were not a valid one.

Preliminary analysis of the curve for dialyzed hemoglobin has shown that the interpretation of these data is by no means as simple as that with undialyzed solutions (if the salt effect is neglected). Log  $y$  against log  $p$  plots (Ferry and Green (20)) are no longer straight lines.

The fact that bicarbonate and phosphate have the greatest



effect may have a physiological significance in that these salts increase the oxygen tension in the blood when it is in closest contact with respiring tissue.

#### SUMMARY

The addition of salts ( $\text{NaCl}$ ,  $\text{KCl}$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ,  $\text{NaH}_2\text{PO}_4$ ,  $+\text{Na}_2\text{HPO}_4$ ,  $\text{NaHCO}_3 + \text{CO}_2$ ) at different concentrations to dialyzed human hemoglobin inhibits the oxygenation of hemoglobin. The inhibiting effect increases with the nature of the salts in the order described. In some cases this inhibiting effect is very probably not due to an ionic strength effect but to the combination of anions with hemoglobin.

The spectra of hemoglobin and oxyhemoglobin in the ultraviolet and visible regions of the spectrum up to  $7700 \text{ \AA}$ . have been determined.

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## COMPLEMENT ACTIVITY AS INFLUENCED BY CERTAIN CHEMICAL AGENTS\*

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In preceding papers (1), it has been shown that a direct correlation exists between the concentration of ascorbic acid and the complementary activity of guinea pig serums. Serums with optimum quantities of ascorbic acid added to them also show a distinct rise of activity and increased stability. Furthermore, it has been noted that ascorbic acid does not reactivate the end- or the mid-piece of complements. However, an increased activity is noted when mid- and end-pieces are combined.

Complement denatured by yeast improves by the addition of ascorbic acid, while  $\text{NH}_3$ -treated complement does not show reactivation. Aeration of complement reduces its activity, which may be restored by ascorbic acid.

Valley (2), in 1928, showed that "spontaneous" inactivation of complement is, at least partially, a reversible reaction and succeeded in reactivating complement with sodium hydrosulfite. Gordon and Thomson (3), in 1933, pointed out that iodides and thiocyanates behaved differently from sodium and potassium chlorides, nitrates, and bromides. Iodides and thiocyanates in small doses (just in excess of the inhibitory dose) acting for 2 hours produced an inhibition that was largely or entirely irreversible on dilution.

These observations are all of obvious significance and suggest the possibility that the effect may be largely attributable to reversible chemical actions upon certain definite groups, as shown

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by Shwachman, Hellerman, and Cohen (4), in 1934, in their excellent studies on the reversible inactivation of enzymes and pneumococcus hemolysin.

Accordingly, a study was planned to investigate the reactivation of complement of vitamin C-deficient guinea pigs, aged guinea pig complement, aerated complement, and complement treated with various oxidants, reductants, and components of complement.

### *Method*

The same method of complement titration (initial hemolysis) described in previous studies was followed. Serums showing no initial hemolysis in doses of 0.1 cc. (of a 1:30 dilution) were considered inactive. It may again be stated that the determination of the point of initial hemolysis in complement titration is a most accurate measure of its activity. In order to clarify the data in this study, the results are expressed on a percentage basis and the original titer of the serum considered as 100 per cent active. Complement was daily obtained from the carotid artery or by cardiac puncture of normal stock guinea pigs. All the reagents were carefully prepared, standardized, and dissolved in M/15 phosphate buffer of pH 7. Glassware was free of heavy metals, and the water triple glass-distilled.

The concentrations of oxidants and reductants are found in Tables I and II.

All the controls of the reagents used showed no hemolytic action by themselves. The experiments were repeated and were strictly reproducible.

### EXPERIMENTAL

Complement from vitamin C-deficient guinea pigs (24 days), aged complement (8 and 22 days at 3°), and complement inactivated by aeration were incubated at 37° for 30 minutes with the reductants indicated and immediately titrated. In the aeration experiments the serum was covered with sterile mineral oil. It was noted that the period of aeration necessary for controlled oxidation varied with the different serums (3 to 7 hours). The results are summarized in Table I.

Fresh complement was then inactivated with iodine,  $H_2O_2$ ,

quinone,  $\text{Cu}_2\text{O}$ , and  $\text{C}_6\text{H}_5\text{HgCl}$ , and subsequently treated with the following reductants:  $\text{H}_2\text{S}$ , KCN, ascorbic acid, and various fractions of fresh complement. The quantities of oxidants and reductants employed are found in Table II. The same procedures as described above were followed. Table II gives the results obtained.

It is evident from this summary that complement may be reversibly inactivated by various oxidants under controlled conditions. Prior to each experiment the smallest amounts of oxidants

TABLE I

*Reactivation of Complement from Vitamin C-Deficient Guinea Pigs, and Complement Inactivated by Aeration and by Prolonged Standing in Refrigerator*

The results are expressed in per cent activity.

Serum	Serum from vitamin C-deficient guinea pigs 24 days on scorbutogenic diet	Serum inactivated by 4 hrs. aeration	Serum inactivated or weakened by aging	
			8 days	22 days
A. Original (1:30) control. ....	100	100	100	100
B. After inactivation (1:30) .....	44	0	50	0
1 cc. (B) 1:20 + 0.5 cc. $\text{H}_2\text{S}$ (water-saturated).....	80	83	100	66
1 cc. (B) 1:20 + 0.5 cc. KCN (0.002 N)....	44	30	40	0
1 " " 1:20 + 0.5 " $\text{Na}_2\text{S}_2\text{O}_4$ (0.2%)....	57	62	50	0
1 " " 1:20 + 3 mg. ascorbic acid + 0.5 cc. Brooks' solution.....	80	50	80	0

and the shortest period of incubation should be determined because the serums show marked variability in their resistance to oxidation. However, little variability was noted in the amounts of the reductants required.

Quinone-inactivated serum showed marked resistance to reactivation. This may be due to specific actions other than simple oxidation.

Since the inactivated complement may be reactivated by all the known components, it can be assumed that the oxidants employed have no apparent effect on these fractions. The effect

must be oxidative in character and probably involves either thiol groupings, ascorbic acid, or lipids.

For inactivations by  $\text{Cu}_2\text{O}$  and  $\text{C}_6\text{H}_5\text{HgCl}$ , the amounts indicated in Table II were shaken with complement for 5 minutes, allowed to stand for 45 minutes at room temperature, and centrifuged.

TABLE II

*Controlled Inactivation of Complement by Oxidants and Metal Compounds with Subsequent Reduction and Reactivation*

Iodine solution: Each cc. contained 0.01 N  $\text{I}_2$  + 0.0075 N KI in M/15 phosphate buffer of pH 7.

$\text{H}_2\text{O}_2$  solution: 1 cc. of superoxol + 9 cc. of M/15 phosphate buffer of pH 7.

Quinone: 0.001 N in M/15 phosphate buffer of pH 7.

Incubation period, 30 minutes.

The results are expressed in per cent activity.

	1 cc. 1:20 complement + 0.117 cc. 0.01 N $\text{I}_2$	1 cc. 1:20 complement + 0.11 cc. 3 per cent $\text{H}_2\text{O}_2$	1 cc. 1:20 complement + 0.33 cc. quinone	1 cc. 1:20 complement + 30 mg. $\text{Cu}_2\text{O}$	1 cc. 1:20 complement + 25 mg. $\text{C}_6\text{H}_5\text{HgCl}$
A. Control (normal).....	100	100	100	100	100
B. After inactivation.....	0	0	0	0	0
1 cc. (B) 1:20 + 0.5 cc. $\text{H}_2\text{S}$ (water-saturated).....	75	83	0	75	100
1 cc. (B) 1:20 + 0.5 cc. 0.002 N KCN..	0	45	0	75	100
1 " " 1:20 + 3 mg. ascorbic acid + 0.5 cc. Brooks' solution.....	60	33	0	0	0
1 cc. (B) 1:20 + 0.5 cc. 1:10 $\text{NH}_4$ -treated complement.....	75	75	0	85	100
1 cc. (B) 1:20 + 0.5 cc. 1:10 yeast-treated serum.....	85	100	0	100	100
1 cc. (B) 1:20 + 0.5 cc. 1:10 heat-treated serum (56°, 30 min.).....	75	75	0	75	100

## DISCUSSION

In view of our previous studies showing the importance of ascorbic acid in complement function, it can now be pointed out that complement can be effectively oxidized and reduced, provided that all oxidations and reductions be controlled. Uncontrolled oxidations lead to irreversibility.

The only conclusion that can be drawn from the literature is that complement consists of a number of components (4) acting in sequence. On this basis it is at present impossible to state that the oxidation-reduction control is exerted on a hypothetical complement molecule or on an equally hypothetical auxiliary substance needed for its final activity. Our observations thus far have proved the existence and necessity of some auxiliary agent or agents like ascorbic acid and possibly glutathione, factors that may be called fifth and sixth components in terms of the old school.

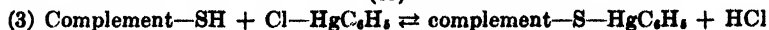
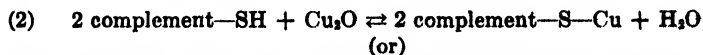
Considering the comparatively low concentrations of reductants in the complement dilutions employed, the necessity of relatively large amounts of oxidants or metals needed to inactivate, and the likewise large amounts of reductants required for reactivation, the assumption that oxidation-reduction effects may act on the "whole complement" itself rather than on a supplementary substance alone is not entirely without foundation.

*In vivo*, under normal conditions, the reductants occur in sufficient amounts adequate to control the system. Such, however, is not the case in vitamin C-deficient animals. Reduction of complement of these deficient animals by means of  $\text{H}_2\text{S}$ ,  $\text{Na}_2\text{S}_2\text{O}_4$ , or ascorbic acid rapidly improves the titer of their serums, indicating a possibility of oxidation *in vivo* during vitamin C deficiency. On standing, complement gradually loses its activity. Here again, the phenomenon appears to be of an oxidative type, since reduction will restore the activity of the weakened complement provided that the oxidation is not continued beyond the point of reversibility.

Iodine, quinone,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2$  oxidize sulfhydryl to disulfide. On this basis the most probable reaction in a complement system would seem to proceed as follows:

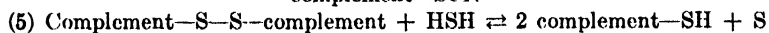
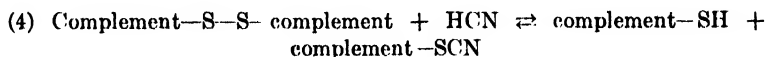


$\text{Cu}_2\text{O}$  and  $\text{C}_6\text{H}_5\text{HgCl}$  are known to be almost specific for thiol compounds and form mercaptides. The nature of these reactions may be as follows:



Since the filtrates of complement treated with  $\text{Cu}_2\text{O}$  and  $\text{C}_6\text{H}_5\text{HgCl}$  showed full complementing activity in latent form, the possibility of adsorption phenomena is thereby eliminated.

In the case of the reductants, like  $\text{HCN}$  and  $\text{H}_2\text{S}$ , we may assume that the reactions occur as follows:



From expressions (4) and (5) it is clear that  $\text{H}_2\text{S}$  could theoretically cause regeneration of all the sulfhydryl originally present before oxidation, whereas  $\text{HCN}$  can regenerate only one-half. This is roughly supported by our data.

$\text{H}_2\text{S}$  had a marked reactivating effect on all oxidized complement and complement treated with heavy metals.  $\text{KCN}$  seemed to have but slight effect on iodine-inactivated complement. The effect of ascorbic acid was comparable to that of  $\text{H}_2\text{S}$ . Complement inactivated by  $\text{Cu}_2\text{O}$  was reactivated by  $\text{KCN}$  and not by ascorbic acid. It can therefore be assumed that  $\text{KCN}$ , and not ascorbic acid, possesses the ability to regenerate sulfhydryl groups from their metallic derivatives in this case.

The fact that iodine- and  $\text{Cu}_2\text{O}$ -inactivated serums could be reactivated with heated serum, third and fourth components, indicates that no known component is affected by these agents. This would seem to prove that these reagents operate on a different component or components, or that only a typical oxidation of the complex occurs.

A great many details remain to be studied. The important observation is that complement is controlled by oxidation and reduction and greatly resembles certain hydrolytic enzymes.

#### CONCLUSIONS

1. Complement obtained from scorbutic guinea pigs can be reactivated by various reductants, like  $\text{H}_2\text{S}$ , ascorbic acid, and sodium hydrosulfite.

2. Aged complement not more than 22 days old can be reactivated by  $\text{H}_2\text{S}$ , while 8 day-old complement can be reactivated by either  $\text{H}_2\text{S}$  or ascorbic acid.

3. Normal complement can be reversibly inactivated by a variety of oxidizing agents provided that the inactivation be controlled (iodine,  $\text{H}_2\text{O}_2$ , quinone, and  $\text{O}_2$ ). The same holds true for  $\text{Cu}_2\text{O}$  and  $\text{C}_6\text{H}_5\text{HgCl}$ . Complement inactivated by these agents can be regenerated by various reductants like  $\text{H}_2\text{S}$ , ascorbic acid, etc.

4. Normal complement inactivated by controlled oxidation with the agents mentioned above can also be regenerated by heat-inactivated complement and by complement lacking the third or the fourth components.

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## COMPLEMENT FUNCTION AS INFLUENCED BY SZENT-GYÖRGYI'S HEXOXIDASE\*

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In the course of work on controlled oxidation and reduction of complement of guinea pig serums, it was shown that the activity probably depended on certain oxidation-reduction systems (1). The reducing function of ascorbic acid was clearly demonstrated.

Both ascorbic acid and glutathione (GSH) are the most active reductants of living tissues and form oxidation-reduction systems which are chemically reversible.

Hopkins and Morgan (2) have recently shown mutual relations displayed by glutathione (GSH) and ascorbic acid in the presence of a hexoxidase extracted from florets and the central white stalks of the cauliflower. The enzyme was described by Szent-Györgyi (3, 4) in 1928 and this author showed that the enzyme oxidizes ascorbic acid reversibly. He remarked that, "Glutathione remains unoxidized in the presence of hexoxidase. If, however, hexuronic acid is present, the glutathione is oxidized; the hexuronic acid plays the rôle of catalyst; it is oxidized by the enzyme and reduced by glutathione."

Hopkins and Morgan concluded that when ascorbic acid and glutathione are together in the presence of the hexoxidase the glutathione wholly protects the vitamin from oxidation and that only when the glutathione has practically disappeared does oxidation of ascorbic acid begin.

They have also pointed out that oxidized ascorbic acid may be

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reduced in the presence of the enzyme 5 times as fast as the rate of oxidation.

In view of the relationship between ascorbic acid and complement function, it was deemed of interest to determine the effect of Szent-Györgyi's enzyme upon complement activity.

### *Method*

The enzyme strength was not standardized because the same preparation was used for each set of experiments. The hexoxidase employed was prepared by three different methods. Florets and central white stalks of cauliflower were the source of the enzyme and only home-grown fresh cauliflower was used throughout.

The first preparations were made by grinding the florets and stalks in a meat chopper and extracting the juice by pressure and through double thickness of gauze. The material was centrifuged four times and the supernatant liquid decanted. The pH was adjusted to 7. By this method the enzyme solution was highly colored and slightly turbid.

The enzyme was also prepared by the method of Hopkins and Morgan in which the juice was expressed by means of a screw press, filtered through linen, and centrifuged. A clear, slightly colored liquid preparation was the result.

Finally, the enzyme was concentrated and purified by the method of Szent-Györgyi (4) (1931). The expressed juice was saturated with  $(\text{NH}_4)_2\text{SO}_4$  and the precipitate resuspended in M/15 phosphate buffer of pH 5.9. This was allowed to stand in the refrigerator overnight, centrifuged, and the supernatant liquid adjusted to pH 7. All the preparations, in the amounts employed, showed no hemolytic effects by themselves. The enzyme can also be concentrated and purified with  $\text{MgSO}_4$ .

The above preparations were dialyzed for a period of 48 hours in running tap water, tested for the presence of  $\text{SO}_4$  ions, and brought up to isotonicity with  $\text{NaCl}$ .

All the reagents used were prepared with triple glass-distilled water and the glassware was free of heavy metals. The same method of complement titration described in preceding articles has been followed and the results are given in per cent of activity.

## EXPERIMENTAL

Fresh guinea pig complement was diluted (1:10) with Brooks' solution and varying amounts (0.2 to 2 cc.) of the hexoxidase preparation were added to 1 cc. portions of the diluted complement. The mixtures were allowed to incubate at 37° for 1 hour, after which time Brooks' solution was added to make a final dilution of 1:30. The complement was then titrated as described elsewhere (1).

*Action of Undialyzed Hexoxidase on Complement*—An experiment showed divergent results in the case of the effects of crudely ground and pressed cauliflower juice. 0.2 cc. of the ground and extracted juice lowered complementary activity, while larger amounts (1.2 cc.) allowed full activity. The pressed juice in amounts ranging from 0.2 to 2 cc. reduced complementary activity. A maximum of 60 per cent reduction of activity was observed when 1.2 to 2.0 cc. of the enzyme were employed. It may be assumed that in the ground preparations certain cellular substances were carried over, acting as inhibitors or reductants.

*Action of Dialyzed Enzyme on Complement*—The dialyzed enzyme inactivated complement in comparatively small amounts. 0.2 cc. of the ground and dialyzed enzyme caused 60 per cent loss of activity. When purified by the method of Szent-Györgyi, it gave a 45 per cent loss of complement titer. Total inactivation was observed with 0.4 cc. of these preparations. No reduction effect was found in the case of the dialyzed enzyme.

It is of interest that Hopkins and Morgan observed no difference in the ability of the undialyzed or dialyzed extracts to oxidize ascorbic acid, but found that the rate of reduction of the vitamin was much more accelerated in the undialyzed than in the dialyzed preparations. They also stated that the rate of reduction is less than the rate of oxidation in the dialyzed preparations.

*Thermolability of Hexoxidase*—The hexoxidase prepared by the method of Szent-Györgyi was subjected to temperatures of 56°, 66°, and 100° respectively for 30 minutes. The heat-treated preparations (1 cc.) were then incubated with fresh complement (2 cc. of a 1:20 dilution) for 1 hour. The hexoxidase was almost completely inactivated at 56° and totally at 66°. The fraction of the juice capable of complement inactivation is therefore thermolabile.

*Effect of H<sub>2</sub>S, Ascorbic Acid, and Various Components of Complement on Hexoxidase-Inactivated Complement*—Complement was inactivated with the purified hexoxidase, and 1 cc. samples of a 1:20 dilution of the inactivated complement were treated respectively with 0.5 cc. of H<sub>2</sub>S water, 1 mg. of ascorbic acid per cc., and a combination of these two, 0.5 cc. (1:10) of heat-inactivated complement, 0.5 cc. (1:10) of yeast-inactivated complement, and 0.5 cc. (1:10) of NH<sub>3</sub>-inactivated complement. The mixtures were incubated at 37° for 30 minutes.

The hexoxidase-inactivated complement was restored 33 per cent by the addition of H<sub>2</sub>S water, 37 per cent by ascorbic acid, and a combination of these two agents gave a 43 per cent return of activity. The inactivated complement was markedly reactivated by the various components of complement. NH<sub>3</sub>- and yeast-treated complements yielded each 66 per cent reactivation and heat-inactivated complement gave a 71 per cent return. It was, however, noted that in uncontrolled oxidation (incubation period of 3 hours) complement may be irreversibly changed so far as reactivation by H<sub>2</sub>S, ascorbic acid, and the third component (yeast-treated complement) is concerned. Heat- and NH<sub>3</sub>-treated complements gave results similar to those observed under controlled oxidations; namely, 66 and 71 per cent returns. It can therefore be assumed that in this type of uncontrolled oxidation the third component is affected.

#### DISCUSSION

It has been demonstrated that complement activity is dependent on a state of reduction for its optimum effect. It is therefore self-evident that if an agent could be found which would act specifically on certain oxidation-reduction systems in the tissues and blood, the nature of the mechanism would be greatly clarified.

Szent-Györgyi (4) in 1931, Tauber and coworkers (5) in 1935, and Hopkins and Morgan (2) in 1936 have observed that hexoxidases are highly specialized in their activity. If such is the case, it can be assumed that the purified enzyme employed in this study specifically acted on ascorbic acid and glutathione (GSH).

Although the findings in this work are of a different nature from those described by Hopkins and Morgan, the final results appear to be comparable. These authors noted that in undialyzed preparations of the hexoxidase, the ability of the hexoxi-

dase to oxidize ascorbic acid is the same as that of a dialyzed preparation, while the reductive ability of the latter is considerably altered.

The results of the present study indicate that the undialyzed and dialyzed preparations are markedly different in their actions on guinea pig complement. In all probability the undialyzed hexoxidase has the power to keep the thiol groupings and ascorbic acid in a reduced state, owing to the fact that the reduction of these agents must be a more rapid process than the direct enzymic oxidizing action. In the dialyzed hexoxidase, the reducing action is lost and the direct enzymic oxidizing action is brought into full play.

The observation that the complement-inactivating fraction of the plant extracts is thermolabile rules out the possibility of some inorganic or other thermostabile substances being anticomplementary.

Of interest is the finding that in uncontrolled inactivation with the dialyzed hexoxidase, the third component is destroyed. Only two other substances are thus far known to destroy the third component; namely, yeast and cobra venom.

#### SUMMARY

1. Guinea pig complement is reversibly inactivated by dialyzed hexoxidase prepared from the florets and the white stalks of cauliflower.

2. The inactivating portion of the hexoxidase is thermolabile.

3. In controlled inactivation by dialyzed hexoxidase,  $H_2S$  and ascorbic acid partially reactivate the hexoxidase-treated complement. All the known components of complement markedly restore the activity of complement treated with the purified hexoxidase.

4. In uncontrolled inactivation by dialyzed hexoxidase, the third component is destroyed.

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# CHEMICAL AND IMMUNOLOGICAL STUDIES OF THE EFFECTS OF RADIANT ENERGY AND OF OXIDATION ON CRYSTALLINE UREASE

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The present investigation was undertaken to secure information as to the chemical and immunological nature of the effect of ultra-violet light and of oxidation on crystalline urease.

Solutions of urease were subjected to irradiation and to oxidation. These solutions were then treated with various reducing agents and compared, also, as to their antigenic characters.

It will be seen from the data and charts presented herein that the nature of the change produced is different in the two cases.

Jacoby (1933) studied the effect of various heavy metals on urease and its reactivation by KCN. Perlzweig (1932) suggested that sulfhydryl groupings might be responsible for the activity of urease. Sumner and Poland (1933) showed the presence of a sulfhydryl group in the urease molecule. Hellerman, Perkins, and Clark (1933) subjected urease to oxidation and reduction and demonstrated the reversibility of the reaction. Pincussen (1923), while studying the effect of ultraviolet light on urease, observed that the enzyme was destroyed by irradiation and that boiled urease has a reactivating effect. Collier and Wasteneys (1932) noted that non-crystalline urease was inactivated by ultraviolet light and by infra-red rays. Tauber (1930), using a crystalline preparation of urease, found that ultraviolet light inhibits urease and that this inactivation is inversely proportional to the distance of the sample from the arc. Kubowitz and Haas (1933) determined the absorption and destructive spectra and observed that the two coincided.

So far as we have been able to find, the literature yields no



information on the effect of various reducing agents on irradiated urease. It was therefore deemed of interest to study the effect of various reductants on irradiated urease and the action of ultraviolet light on urease in an oxygen-free menstruum. The irradiated and oxidized enzymes were further investigated for their immunological behavior.

### *Material and Methods*

#### *Chemical*

*Crystalline Enzyme*—Sumner's (1926) method for the crystallization and recrystallization was followed throughout these studies. Extreme care was taken in preparing all reagents and all glassware was freed of traces of heavy metals. Under these conditions crystallization was easily accomplished. No protective colloid, such as gum arabic, was used, since only freshly prepared urease crystals were employed.

The enzymic activity was measured in units, 1 unit being the amount necessary to hydrolyze 1 mg. of N from a 3 per cent urea-buffer solution in 5 minutes at a temperature of 20°. This method is essentially the same as that advocated by Sumner.

*Reagents*—H<sub>2</sub>S water was obtained by bubbling H<sub>2</sub>S through triple distilled water until saturated. KCN and sodium hydro-sulfite solutions were prepared from Merck's Blue Label Reagents dissolved in triple glass-distilled water. The standard Nessler's solution and 3 per cent urea-buffer solution employed were the same as used by Sumner. Acetone was triple distilled from fused calcium chloride and soda lime. The buffers (phosphates) were made up according to Sørensen.

*Apparatus*—Irradiation of the crystalline urease was carried out with a carbon arc lamp which was kindly placed at our disposal by the National Carbon Company of Cleveland. It proved very satisfactory. The C type of carbon was the most satisfactory. The materials were exposed in quartz tubes.

For the oxidation of the enzyme we made use of an aeration apparatus similar to that used in urea determinations.

*Methods of Inactivation and Reactivation*—Two methods of inactivation were used: radiant energy and oxidation. Crystalline urease of a dilution of 10 units per cc. was irradiated by the use of a C carbon, a rich source of ultraviolet rays, for various

periods of time and controls of urease in plain glass tubes were simultaneously exposed so as to rule out possible deviations through heat denaturation. The temperature of the laboratory was 20°. Immediately after irradiation the activity of the enzyme was determined, since treated enzymes that were allowed to stand at room temperature showed a continued decrease of activity.

Anaerobic irradiation was performed by repeated exhaustion of all air from the tubes and the tubes were refilled with oxygen-free nitrogen. All the tubes were tightly sealed.

Solutions of urease were oxidized by bubbling air through the solution for various periods of time. A trace of  $\text{Cu}_2\text{O}$  was added to accelerate inactivation. The air was bubbled through slowly, so that no defoaming reagent was needed. The temperature was kept at about 25°. Inactivation through iodine was accomplished with 0.002 N iodine.

The reductants were added to the various inactivated urease solutions and allowed to stand at 25° for 15 minutes, after which time the activity was immediately determined.

### *Immunological*

*Urease Preparations*—Recrystallized urease was used throughout, since only an antigen of the highest purity can be relied upon. No preservative was added and fresh solutions were prepared for each injection. The injections were made by the intraperitoneal route. Just before the injection the solutions were made isotonic with NaCl and the volume of the solution was kept as small as possible. Irradiated, oxidized, and boiled urease containing originally the same number of units was treated as above and used for immunization.

*Animals*—Medium sized rabbits were employed. Before injections were begun the serum of each animal was tested for the presence of natural antibodies against irradiated, oxidized, boiled, and undenatured urease. All animals were kept on a mixed diet and all food was withheld for a period of 24 hours prior to each bleeding.

*Method of Immunization*—Weekly injections were given over a period of about 10 weeks. The amounts of urease injected ranged from 5 units to a final dose of 500 units.

*Tests for Immunity*—Precipitation (ring test), determination of the antiurease titers of the serums, and the survival of the animals following the injection of massive doses of the active enzyme were used as tests for immunity. 3 cc. of blood were drawn into the paraffin-coated centrifuge tubes before each injection. The separated clear serum was diluted (1:32) with 0.85 per cent saline. The antigen (urease) was diluted so that 1 cc. contained 5 units.

The ring test was performed in Hektoen tubes which were thoroughly cleaned with dichromate solutions and rinsed with double distilled water. 1 drop of the urease was layered on 1 drop of serum. The tubes were kept for 1 hour at 37°.

The test for antiurease was essentially the same as described by Kirk and Sumner (1931-32) 1 unit of antiurease being the amount that will neutralize 1 unit of urease. This test is the most delicate, since the neutralizing antibodies exert their effect upon the activity of the enzyme and can therefore be measured.

The test for resistance against massive doses of the urease was accomplished by the intraperitoneal injection of 500 units (10 times the minimum lethal dose) of urease into each test animal 3 weeks after the last injection was made.

*Reactivation of Oxidized Urease with Tissue Extracts and Blood*—The tissues (liver and kidneys) were obtained from normal rats killed by a blow. The tissues were freed of all extraneous materials and ground with washed sand. The ground up mass was then extracted with constant stirring with equal parts of triple distilled water. After a 10 minutes extraction, the mass was centrifuged at high speed. 3 cc. of the supernatant fluid were added to different samples of oxidized urease and allowed to incubate at 37° for 30 minutes. The emulsion was then brought to a temperature of 20° and 1 cc. of Sumner's 3 per cent buffered urea solution was added. This was kept at 20° for 5 minutes, and 2 cc. of freshly prepared 10 per cent metaphosphoric acid were added. Precipitated proteins were centrifuged off. The supernatant liquid was then nesslerized and the ammonia N determined. Controls were run at the same time on the tissue extracts for  $\text{NH}_3$  and urea. Recovery tests by the above methods showed little error.

The same procedure was followed in the case of whole blood. 3 cc. of citrated blood were employed instead of tissue extracts.

## EXPERIMENTAL

Preliminary studies showed that crystalline urease can be reversibly inactivated by oxidizing agents, such as iodine, and also by aeration. After controlled inactivation,  $\text{H}_2\text{S}$  water gave 80 per cent reactivation and KCN showed 60 per cent reactivation.

The influence of ultraviolet light on crystalline urease and the effect of  $\text{H}_2\text{S}$  and of KCN on the irradiated products were then studied. Irradiation was carried out at a distance of 45 cm. from the carbon arc. No denaturation by heat was noticed, as controls showed no inactivation of the enzyme.

Samples were taken at 5, 10, 15, 20, 25, 30, and 60 minute intervals. The samples were then incubated with 2 cc. of  $\text{H}_2\text{S}$  water, and 1 cc. of 0.05 N KCN respectively. Activity was immediately determined after 15 minutes at 25°.

Crystalline urease was rapidly and permanently destroyed by irradiation by ultraviolet light. The extent of inactivation was about 35 per cent with 10 minutes irradiation, 70 per cent in 20 minutes, and 90 per cent in 30 minutes. The process is not reversible, since the reducing agents had no apparent reactivating effect.

Crystalline urease was sealed in quartz test-tubes and connected to a 2-way stop-cock. The solutions were repeatedly exhausted of all air, and subsequently refilled with  $\text{O}_2$ -free nitrogen. The same procedures employed before were then followed. It was found that the course of inactivation was substantially unaffected by the presence of air, potassium cyanide, or hydrogen sulfide. It was again impossible to reactivate the irradiated products with  $\text{H}_2\text{S}$  water.

The second series of experiments conducted was purely immunological. The results of this series are given in Tables I to III.

Table I indicates the results of the antigenic properties of the various urease compounds by the precipitin (ring test) method, and the method of Sumner for measuring the amount of anti-urease evolved.

From these results it is seen that the oxidized urease and the original active preparation yielded the same antigenic response, and immunity was produced by both products. It seems that the irradiated urease and the boiled urease were unable to produce a

specific antiurease. It is of interest to note the rapidity of the formation of antiurease in the animals.

The resistance of the experimental animals to 500 units of urease, 3 weeks after the final injection, was striking. The animals which had been inoculated with oxidized and non-oxidized urease suffered no apparent ill effects and remained well, while

TABLE I

*Results of Antigenic Properties of Various Urease Compounds*

Rabbits were injected after each bleeding with doses of crystalline urease ranging from the initial dose of 5 Sumner units to a final dose of 500 units. Equivalent amounts of oxidized, boiled, irradiated urease were given to designated animals.

Rabbit No. ....		Boiled urease*	Urease		Irradiated urease				Oxidized urease			
			1	2 + 7 (average)	3		4 + 9 (average)		5		6 + 8 (average)	
Date	Units injected	Precipitin ring test	Antiurease	Precipitin ring test	Antiurease	Precipitin ring test	Antiurease	Precipitin ring test	Antiurease	Precipitin ring test	Antiurease	Precipitin ring test
1937			units†		units		units		units		units	
Jan. 24	5	—	0	—	0	—	0	—	0	—	0	—
Feb. 1	15	—	3.1	++	0	—	0	—	0	+	0	—
" 9	40	—	4.3	+++	0	—	0	—	0	+	1.2	++
" 23	75	—	7.1	++++	0	—	0	—	1.0	+	1.1	+++
Mar. 6	150	±	10.1	++++	0	±	0	±	1.9	++	3.6	+++
" 19	300	±	11.9	++++	0.8	+	1.2	±	4.5	+++	7.7	++++
" 23	500	+	12.8	++++	1.2	+	1.0	+	6.9	++++	9.1	++++
Apr. 14†	500	§	Alive		Dead		Dead		Alive		Alive	

\* Animals injected with boiled urease produced no antiurease.

† 1 unit of urease is neutralized by 1 unit of antiurease.

‡ Crystalline urease.

§ Dead.

the animals previously injected with the boiled and the irradiated enzyme died of urease poisoning (ammonia poisoning) within 2 to 3 hours after the injection.

Table II shows the results of cross-precipitation of the various enzyme preparations and their homologous serums. The data are again self-evident. It is seen that the specificity of the irradi-

ated enzyme to react *in vitro* with active crystalline urease anti-serum is not lost, while the oxidized urease seems to have lost this power. It is of great interest to note that the ability of the various antigens to induce a precipitate in non-homologous serums is divergent. The possible explanation of these phenomena is given in the discussion.

Table III summarizes the results of studies on the reactivation of oxidized urease with tissue extracts and whole blood.

TABLE II  
*Cross-Precipitin Tests on Different Serums and Urease Preparations*

Antiserums from	Irradiated urease	Oxidized urease	Boiled urease	Urease
Oxidized urease.....	+++	++	—	+++++
Irradiated urease.....	+++++	+	++	±
Boiled urease.....	+	+	+++++	±
Crystalline urease.....	+++	±	±	+++++

TABLE III  
*Effect of Tissue Extracts and Whole Blood on Crystalline, Irradiated, and Oxidized Urease*

Urease	Activity	Rats (2) 3 cc. of tissue extract added	Rabbits (2) 3 cc. of whole blood added
	units	units	units
Mar. 24. Normal.....	5	5.2	5.3
Oxidized.....	1.9	3.95	1.7
Irradiated.....	2.1	1.95	2.35
Oxidized.....	0	1.72	0
Apr. 8. Normal.....	5	5.3	5
Oxidized.....	0	1.78	0
Irradiated.....	1.2	1	1.3

Tissue extracts have a reactivating effect on oxidized urease, while blood has no such action. There is no apparent change in the activity of the irradiated products.

#### DISCUSSION

The question as to whether it is possible to induce immunity with reversibly inactive enzymes and not with irreversibly inactive enzymes is of fundamental importance because the answer

may give us some insight, not only into the antigenic character of these agents, but also some information as to their chemical nature. The answer is beset with several difficulties. First, one cannot be certain that the enzyme has been completely inactivated. Second, the production of immunity to enzymes has been a difficult task. Third, there had been no mechanism for the accurate measurement of immunity to enzymes, until the intensive studies of Sumner and coworkers appeared. Fourth, before the period of crystallization of urease, no *pure* enzyme was available for such a study.

Crystalline urease overcomes all of these difficulties. It has been shown by Sumner that this preparation is ideal for the study of the rôle of enzymes in antibody formation. So, with the aforementioned difficulties overcome, it seemed of great interest to study the production of immunity by different inactive urease preparations.

We have shown that oxidized urease has the capacity to form antiurease in amounts almost similar to that formed by the active crystalline preparations, while the irradiated preparations do not have this property. It will be recalled that in the chemical aspects of these experiments oxidized urease could be reversibly reactivated, while this could not be accomplished with the irradiated enzyme. This fact points to the possibility that the irradiated enzyme is denatured and that a marked change has occurred in its protein molecule. It can also be assumed that no such change took place in the oxidized enzyme, and that in all probability a change occurred in a side chain or in some chemical linkage in the compound. The most reasonable assumption is the one offered by Hellerman, showing that the  $\text{—SH}$  groupings are oxidized to  $\text{—S—S—}$  groupings.

Stanley (1936) found no change in the immunological properties of the tobacco mosaic virus after irradiation. As has been mentioned before, the biological method of test for the activity of an enzyme or virus is far less specific than a chemical one. Although virus and enzymes have often been compared and considered similar, recent studies seem to show this to be an error. Poland (unpublished) found that formolized urease yielded no antiurease, while Stanley (1936) and Chester (1936) obtained the same immunological response with tobacco virus that had been

treated with formaldehyde as with normal tobacco virus. It is probable that we are dealing with two separate entities.

Landsteiner (1936) and others have recently shown the parts played by hapten groups and the protein colloids associated with them. The hapten group supplies the specificity but has no apparent antigenicity. The antibody-producing substance is conveyed by the protein body of the molecule. The data obtained by cross-precipitations shows the possibility that the sulfhydryl groupings (or whichever group is oxidized and reduced) may carry the specificity of the compound. It has been shown here that irradiated urease has the ability to precipitate the serums from animals immunized with oxidized and active urease, while oxidized urease has far less ability to do this. It will be noticed also that each enzyme preparation has the capacity to precipitate its homologous serum, thereby demonstrating the presence of an active specific antigenic portion in their individual compositions.

Blumenthal (1936) found that the precipitability of serum albumin is irreversibly decreased after reduction of the disulfide groups, and, as an explanation, the author suggests either participation of the  $-S-S-$  and  $-SH$  groups in the reaction, or a splitting of the antigenic molecule by opening of the  $-S-S-$  linkage.

The fact that oxidized urease can be actively reduced with tissue extracts, and not by blood, points to the mechanism by which the oxidized urease is made specifically antigenic in the animal. This activation can be accounted for by the presence of glutathione in the tissues, which has been shown by Borsook and coworkers to reduce dehydroascorbic acid *in vitro* to the reduced ascorbic acid.

Further studies along this line may throw light on this insufficiently explored field of the specificity of immune reactions.

#### SUMMARY

1. Oxidized urease may be reactivated with reducing agents such as  $H_2S$  and  $KCN$ . This confirms the work of Hellerman.

2. Crystalline urease is rapidly and permanently destroyed by exposure to ultraviolet light. Urease inactivated by irradiations cannot be reactivated by the various reducing agents.



3. Immunologically, urease and oxidized urease produced a similar antibody, while irradiated urease failed to produce an antiurease. Animals immunized with oxidized urease were able to withstand 10 times the minimum lethal dose of active urease, while animals immunized with irradiated urease succumbed.

4. Tissue extracts reactivated oxidized urease, but did not reactivate irradiated urease. Whole rabbit blood did not reactivate oxidized urease.

5. Sulfhydryl groupings appear to enter into the antigenic specificity of the enzyme urease.

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# THE FORMATION OF GLUCOSE-1-PHOSPHORIC ACID IN EXTRACTS OF MAMMALIAN TISSUES AND OF YEAST

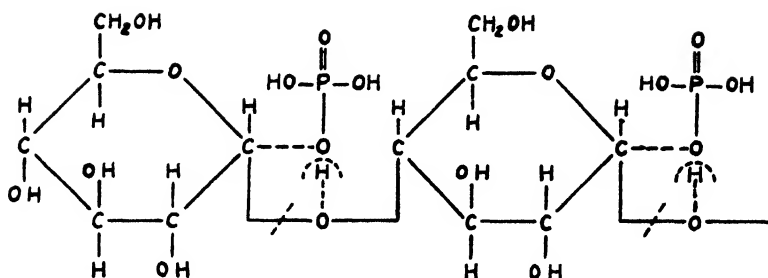
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The initial stages of fermentation of glycogen to lactic acid in muscle have been investigated only recently. It was shown (1, 2) that the glycolytic reactions are initiated by the formation of glucose-1-phosphoric acid from glycogen and inorganic phosphate, an enzymatic process in which adenylic acid acts as coenzyme. The glucose-1-phosphoric acid (1-ester) is converted to glucose-6-phosphoric acid by another muscle enzyme the activity of which is greatly enhanced by magnesium ions. Because of the presence of this enzyme and of magnesium the 1-ester does not accumulate in intact muscle, but it can be made to accumulate in muscle extracts from which magnesium ions have been removed by long dialysis. The isolation of the 1-ester from such extracts and its synthesis have been described (3). Embden ester (an equilibrium mixture of glucose-, fructose-, and probably also mannose-6-phosphate (4)), here referred to as 6-ester, is always present in intact muscle; it is formed from glucose-6-phosphate by an enzyme which Lohmann (5) found in extracts of muscle, brain, liver, kidney, and yeast. With these three reactions the first stages of glycogen fermentation have been completed; then follow the phosphorylation of 6-ester to fructosediphosphate and the reactions described in the Embden-Meyerhof scheme.

It is shown in this paper that the formation of 1-ester from glycogen and inorganic phosphate occurs in extracts of a variety of mammalian tissues and of yeast. The disruptive phosphorylation of glycogen, for which a scheme is presented, may therefore be regarded as a generally occurring process.



The diagram pictures the entrance of inorganic phosphate at the maltosidic linkages. The glycogen molecule is thereby disrupted without the elements of water entering into the reaction. The process has therefore been aptly described by Parnas (6) as "phosphorolysis" to distinguish it from "hydrolysis" of the glycogen molecule. Starch behaves like glycogen, while inulin is not phosphorolyzed.

Neither maltose nor glucose is phosphorylated under conditions under which 1-ester is formed from glycogen by tissue and yeast extracts. It is now known that the phosphorylation of glucose by yeast enzymes is linked with a simultaneously occurring oxido reduction (7). The disruptive phosphorylation of glycogen can occur without simultaneous oxido reduction, a fact which indicates that there are two different enzyme systems involved.

#### EXPERIMENTAL

The extracts of the mammalian tissues mentioned were prepared in the same way as described for skeletal muscle (3). A maceration juice was prepared from dried brewers' yeast according to Lebedeff (8) and was dialyzed for 2 hours.<sup>1</sup>

The muscle extracts were dialyzed for 18 hours, or longer, in collodion sacs against water at 8° and were subsequently stored for several weeks in the refrigerator under toluene without losing much of their activity (3). The extracts of the other tissues could be dialyzed for only 4 to 5 hours, since longer dialysis led to a complete loss of activity, and they could be stored for only 1 or 2 days in the refrigerator without becoming inactivated.

<sup>1</sup> We are indebted to Anheuser-Busch, Inc., for the supply of brewers' yeast.

In these dialyzed extracts the breakdown of glycogen did not progress beyond the hexosemonophosphate stage.

TABLE I

*Formation of Glucose-1- and Hexose-6-Phosphate in Extracts of Various Tissues of Rabbit*

To 6 cc. of extract were added 3 cc. of  $M/3$  phosphate buffer of pH 7.2, 30 mg. of glycogen, and in some cases 2 mg. of adenylic acid and 2 mg. of magnesium (as  $MgCl_2$ ). The mixture was incubated for 1 hour at 24°. Values are given in mg. per 10 cc. of reaction mixture.

Type of tissue	Extract No.	Tissue for 10 cc. extract	1-Ester			6-Ester			Total ester as hexose	Additions
			Hexose	P found	P calculated	Hexose	P found	P calculated		
		gm.								
Skeletal muscle		4.0	1.4	0.3	0.2	2.7	0.4	0.5	4.1	None
			12.8	2.2	2.2	4.0	0.7	0.7	16.8	Adenylic acid
			1.0	0.2	0.2	15.5	2.5	2.7	16.5	" " + Mg
Brain	1	3.1	2.4	0.5	0.4	0.1			2.5	None
			6.4	1.2	1.2	0.1			6.5	Adenylic acid
			6.8	1.3	1.2	0.4			7.2	" " + Mg
	2	3.0	7.0	1.1	1.2				7.0	" "
	3	3.7		1.0			0.7		9.9*	" "
Heart				0.2			1.5		9.9*	" " + Mg
	1	1.9	10.9	2.2	1.9	0.7	0.1	0.1	11.6	" "
			8.3	1.4	1.4	3.6	0.7	0.6	11.9	" " + Mg
	2	1.9	2.4	0.4	0.4	0.6			3.0	None
			10.2	1.8	1.8	0.5			10.7	Adenylic acid
Liver			9.4	1.6	1.6	2.6	0.4	0.4	12.0	" " + Mg
	3	2.0	10.0	1.7	1.8	1.2	0.3	0.2	11.2	" "
	1	2.0	5.8	0.6	1.0				5.8	None
			8.0	1.2	1.4	0.3			8.3	Adenylic acid
			5.7	0.9	1.0	2.1	0.4	0.4	7.8	" " + Mg
Kidney	2	3.3	2.9	0.4	0.5	5.7	1.1	1.0	8.6	" " + "
	3	3.1		0.1			2.0		12.2*	" "
		3.5	0.5			0.9	0.1	0.2	1.4	None
			1.8	0.2	0.3	1.6	0.3	0.3	3.4	Adenylic acid
			1.1	0.1	0.2	1.9	0.3	0.3	3.0	" " + Mg

\* Incubated at 37°. Analysis of P content only, from which hexose content was calculated.

*Determination of 1- and 6-Ester*—After incubating the extracts with phosphate, glycogen, and other additions as indicated in

Table I, they were deproteinized with 2.5 per cent  $\text{HgCl}_2$  in 0.5 N HCl. The 1- and 6-esters were isolated as the water-soluble barium salts as previously described (9). This was done as rapidly as possible because of the instability of the 1-ester in acid solution (3). The 1- and 6-esters were determined as hexose and from this the theoretical amount of organic P to be expected was calculated and was compared with the organic P as actually determined.

The determination of the two esters as hexose is based on the fact that the 6-ester reduces alkaline copper solution, while the 1-ester, which is non-reducing, can be hydrolyzed to glucose and inorganic P by short exposure to acid at  $100^\circ$  under conditions under which the 6-ester is not hydrolyzed. Consequently, the original reducing power of the hexosemonophosphate fraction, determined with the Shaffer-Somogyi reagent containing 1 gm. of KI per liter, corresponds to the amount of hexose in the 6-ester,<sup>2</sup> while the increase in reducing power after 7 minutes hydrolysis in N  $\text{H}_2\text{SO}_4$  at  $100^\circ$  corresponds to the amount of glucose in the 1-ester. In the phosphate analysis, in which the method of Fiske and Subbarow (10) is used, the inorganic P formed during 7 minutes of hydrolysis in N  $\text{H}_2\text{SO}_4$  at  $100^\circ$  corresponds to the P in the 1-ester. The total P after ashing minus the "hydrolyzable" P corresponds to the P in the 6-ester. A small amount of barium phosphate is carried over into the hexosemonophosphate fraction, necessitating a determination of inorganic P before hydrolysis.

### Results

Since several experiments with muscle extracts have been reported (2), only one example is given in Table I, for comparison with the activity of the extracts of other tissues. The extracts of brain, heart, and liver, though less active than those of muscle, showed good phosphorylation, while the kidney extracts were only weakly active. Addition of adenylic acid increased phosphorylation in all cases. The hexosemonophosphate formed (during incubation at  $24^\circ$ ) consisted of a mixture of 1- and 6-esters, with the former predominating. Addition of magnesium resulted in the formation of 6-ester at the expense of 1-ester, but the conversion was not so complete as in the case of muscle extracts,

<sup>2</sup> With this reagent the hexose in the 6-ester has two-thirds the reducing power of glucose.

possibly because the other tissues contain less "conversion enzyme" than muscle. The activity of the conversion enzyme in muscle extracts is greatly increased with a rise in temperature. This was also the case with brain and liver extracts, so that after 1 hour of incubation at 37° mainly 6-ester was present, in the case of liver even if no magnesium had been added. It should be emphasized that the extracts of brain, heart, and liver could be

TABLE II

*Formation of Glucose-1- and Hexose-6-Phosphate in Yeast Extract*

To 6 cc. of extract were added 2 cc. of 1.5 M phosphate and 32 mg. of glycogen. The mixture was incubated for 2 hours at 25°. Values are given in mg. per 10 cc. of reaction mixture.

Extract No.	pH of phosphate added	1-Ester			6-Ester			Total ester as hexose	Additions
		Hexose	P found	P calculated	Hexose	P found	P calculated		
1	4.5	10.8	1.5	1.8	3.1	0.7	0.5	13.9	4 mg. adenosinetriphosphate
	4.5	9.9	1.3	1.7	3.0	0.7	0.5	12.9	4 mg. adenosinetriphosphate + Mg
2	4.5	9.6	1.6	1.7	2.7	0.4	0.5	12.3	4 mg. adenosinetriphosphate
	4.5				1.5	0.3	0.3	1.5*	4 mg. adenosinetriphosphate
3	7.2	4.7	0.7	0.8	8.4	1.3	1.5	13.1	None
	7.2	5.9	0.8	1.0	7.4	1.3	1.3	13.3	2 mg. adenylic acid
	4.5	3.6	0.5	0.6	1.1	0.2	0.2	4.7	None
	4.5	4.1	0.5	0.7	1.3	0.4	0.2	5.4	2 mg. adenylic acid
4	4.5				0.7	0.3	0.1	0.7*	2 " "
	7.2	6.9	1.3	1.2	10.1	1.4	1.7	17.0	None
	7.2	6.0	1.0	1.0	10.7	1.8	1.8	16.7	4 mg. adenosinetriphosphate

\* Substrate glucose instead of glycogen.

dialyzed for only short periods, so that a complete removal of the nucleotides and of magnesium originally present in these extracts was not to be expected.<sup>3</sup>

\* The dialyzed extracts of brain, heart, and liver contained from 0.2 to 0.4 mg. of organic P per 100 cc. and they showed a slight phosphorylation without addition of adenylic acid. In the case of skeletal muscle it was possible to prepare long dialyzed extracts which contained no measurable

The experiments with yeast extracts are shown in Table II. With glycogen as substrate, 1- and 6-esters were formed,<sup>4</sup> while under the same conditions with glucose as substrate, hardly any phosphorylation occurred. The conversion enzyme has its maximum activity at pH 7.5; when the pH of the phosphate added was 4.5 more 1- than 6-ester was present, while the opposite was true when the pH was 7.2. The 2 hour period of dialysis was insufficient for the removal of nucleotides and of magnesium, so that their addition had no effect.

#### SUMMARY

1. Dialyzed extracts of rabbit brain, heart, and liver formed glucose-1-phosphoric acid from glycogen and inorganic phosphate. Kidney extracts showed only weak activity. Addition of adenylic acid increased the ester formation in all cases.

2. Conversion of the glucose-1- to hexose-6-phosphoric acid upon addition of a magnesium salt was less complete in the extracts just mentioned than in muscle extracts.

3. Dialyzed extracts of brewers' yeast formed glucose-1- and hexose-6-phosphoric acid from glycogen and inorganic phosphate under conditions under which glucose was not phosphorylized.

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amounts of organic P and which were unable to phosphorylize without addition of a nucleotide, as shown in the following paper.

<sup>4</sup> Application of the method for the isolation of hexosemonophosphate to the unincubated yeast extracts gave a barium salt fraction which showed some reducing power and contained from 0.2 to 0.4 mg. of organic P per 10 cc. of reaction mixture. The values shown in Table II have been corrected for reducing power and organic P originally present.

## THE ACTION OF NUCLEOTIDES IN THE DISRUPTIVE PHOSPHORYLATION OF GLYCOGEN

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It has been shown previously (1, 2) that addition of adenylic acid accelerates very markedly the formation of glucose-1-phosphoric acid (1-ester) from glycogen and inorganic phosphate in washed muscle residue and in dialyzed muscle extract. Parnas and Mochnacka (3) reported later that not only adenylic acid but also its deamination product, inosinic acid, has an accelerating effect on the esterification of inorganic phosphate in dialyzed muscle extract. This was confirmed and it was shown that inosinic acid, though less active than adenylic acid, gives the same first product of esterification; namely, 1-ester. The present experiments were undertaken in order to investigate more thoroughly the rôle of adenylic, inosinic, and adenosinetriphosphoric acid in the disruptive phosphorylation of glycogen.

### EXPERIMENTAL

For the purpose of this investigation it was found necessary to use muscle extracts which were inactive without addition of nucleotides and which were free of pyrophosphatase and deaminase. The rabbit muscle extracts were prepared as previously described (4); they were kept for 5 hours at 25° and were then dialyzed for 24 hours in thin collodion sacs with inside stirring against running tap water or distilled water at 8°. The extracts were filtered or centrifuged to remove insoluble protein and were stored under toluene in the refrigerator. As a rule such extracts did not form 1-ester unless a nucleotide was added.

Pyrophosphatase interferes by converting adenosinetriphosphate to adenylic acid and deaminase by converting adenylic to inosinic



acid. These two enzymes were present in all freshly prepared extracts. It was found that they generally disappeared after the extracts had stood for 3 weeks in the refrigerator, at which time the phosphorylizing enzyme still retained its activity. Extract 13 in Fig. 1 was such an extract; it formed no ammonia when incubated with adenylic acid, but it still showed a slight pyrophosphatase activity. Another method for removing the enzymes in question consisted in the treatment of freshly prepared extracts with charcoal. To 400 cc. of Extract 15 were added 30 gm. of activated charcoal (Mallinckrodt Chemical Works) and the mixture allowed to stand, with occasional shaking, for 30 minutes at room temperature. After filtration in the refrigerator the charcoal treatment was repeated. Before the charcoal treatment 4.5 mg. of adenylic acid, when incubated with 2 cc. of extract for 1 hour at 37°, yielded 0.187 mg. of ammonia N; calculated, for complete deamination, 0.182 mg. After the charcoal treatment no ammonia was formed. The extent of pyrophosphatase activity after the charcoal treatment is shown in the following experiment. Of 0.173 mg. of pyrophosphate P (added as magnesium adenosinetriphosphate to 2 cc. of extract) 0.167 mg. remained after 1 hour of incubation without creatine and 0.165 mg. with creatine added. (Creatine was added in order to test the extract for the presence of the enzyme which transfers phosphate from adenosinetriphosphate to creatine.) Extract 14 in Fig. 1, which was not treated with charcoal, converted all of the added adenosinetriphosphate to adenylic acid during 1 hour of incubation.

Care was taken to obtain the nucleotides used in this investigation in as pure a condition as possible. Adenylic acid—a commercial sample (Laokoon Company, Lwów, Poland) and a preparation made from rabbit muscle according to Ostern's method (5)—was repeatedly recrystallized from dilute alcohol. P and amino N determinations on a sample dried *in vacuo* gave values close to the theory, the atomic ratio of P to amino N being 1:1.04. Inosinic acid was obtained as the crystalline barium salt from rabbit muscle by Ostern's method (5) and from adenylic acid by deamination with nitrous acid according to Lohmann's method (6). Both samples were recrystallized three times from hot water and after drying *in vacuo* they gave values for P and N corresponding to

those calculated for the anhydrous barium salt. The atomic ratio of P to N was 1:3.95 (theory 1:4). Adenosinetriphosphate was isolated as the barium salt from  $\text{HgCl}_2$  filtrates of rabbit muscle; it gave a ratio of easily to difficultly hydrolyzable P of exactly 2:1. The inorganic P content of the sample, which was originally 2 per cent, did not change during the course of the experiments, showing that no decomposition to adenylic acid had taken place. The barium salts of the nucleotides were converted to the potassium salts before addition to the muscle extracts.

In the present experiments the decrease in the concentration of added inorganic phosphate, determined by the method of Fiske and Subbarow (7) in trichloroacetic acid filtrates of the extracts, was used as a measure of hexosemonophosphate formation. The justification of this procedure is based on comparisons of the amount of inorganic P which disappeared with the amount of hexosemonophosphate P formed, as illustrated in the following example. To 5 cc. of muscle extract were added 1.5 cc. of  $\text{m}/6$  phosphate buffer of pH 7.2, 10 mg. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30 mg. of glycogen, and 1.34 mg. of adenylic acid. 0.5 cc. of the mixture was withdrawn for the analysis of inorganic P before and after incubation (1 hour at  $37^\circ$ ), while 5 cc. were used for the isolation and analysis of the hexosemonophosphate fraction, as described in the preceding paper (8). Per 5 cc. of reaction mixture there were present 5.95 mg. of inorganic P before and 3.37 mg. after incubation, indicating an esterification of 2.58 mg. The hexosemonophosphate fraction contained 2.62 mg. of organic P per 5 cc. When the adenylic acid P added was deducted (0.09 mg. per 5 cc.), 2.53 mg. or 98 per cent of the inorganic P which disappeared was accounted for as hexosemonophosphate.

The results shown in Fig. 1 are representative of a larger number of experiments.<sup>1</sup> Curves A-13 and A-15 show that extracts in which no esterification occurs when incubated with glycogen and inorganic phosphate are activated by the addition of minute amounts of adenylic acid.  $\text{m}/60,000$  adenylic acid, which was the lowest concentration tested, had a noticeable effect and twice this concentration a marked effect on the esterification of

<sup>1</sup> In a previous report (2) the heading of the first column of Table II should read mg. of P per 10 cc. of mixture, P having been omitted through an oversight.

inorganic phosphate.<sup>2</sup> It is assumed that the enzyme which forms 1-ester from glycogen and inorganic phosphate is active only when combined with a nucleotide.<sup>3</sup> Curve A-15 shows that the esterification of inorganic phosphate approaches a maximum with increasing amounts of adenylic acid added. With  $M/1000$

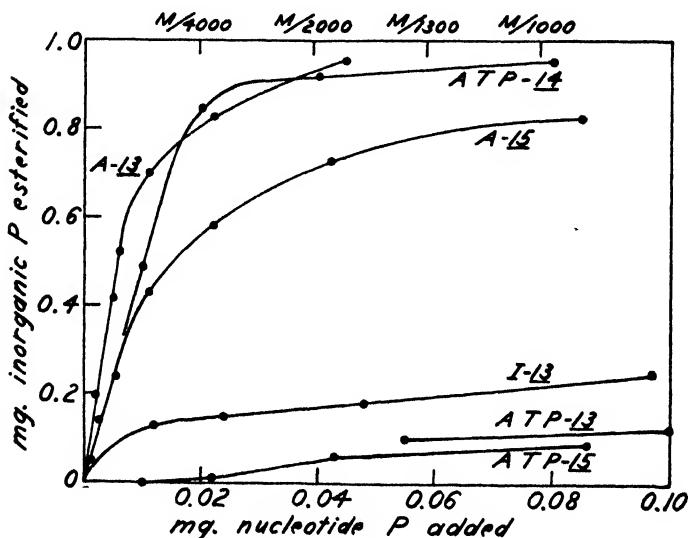


FIG. 1. Effect of nucleotides on esterification of inorganic phosphate in muscle extract. The curves are marked with the number of the extract used and with the type of nucleotide added, A being adenylic, I inosinic, and ATP adenosinetriphosphoric acid. Extract 14, in contrast to the other two extracts, contained pyrophosphatase. 2.5 cc. of an extract-buffer mixture of pH 7.2 contained 1.72 mg. of inorganic P, 0.2 mg. of magnesium, 8 mg. of glycogen, and varying amounts of nucleotides. The samples were incubated for 1 hour at 37°. The abscissa shows the amounts of nucleotide P added (in the case of adenosinetriphosphate this corresponds to one-third of its total P) and the ordinate the amounts of inorganic P esterified, both per 2.5 cc. of reaction mixture.

<sup>2</sup> The initial concentration of inorganic phosphate in these experiments was  $M/45$  or about 3 times that found in intact muscle.

<sup>3</sup> Magnesium is apparently not needed, since esterification of inorganic phosphate occurs in electrodialyzed muscle extracts without its addition. It was added in these experiments because in its presence the acid-stable 6-ester accumulates; for analytical reasons this is preferable to an accumulation of the acid-labile 1-ester.

adenylic acid, which was the highest concentration used, phosphorylation was only 12 per cent greater than with  $M/2000$  adenylic acid.

The curve for inosinic acid (I-13, Fig. 1) does not show the steep rise which is characteristic for adenylic acid and even with  $M/400$  inosinic acid (or 2 times the highest concentration shown in Fig. 1) there was hardly any further increase in esterification of inorganic phosphate. In an experiment with Extract 15, not shown in

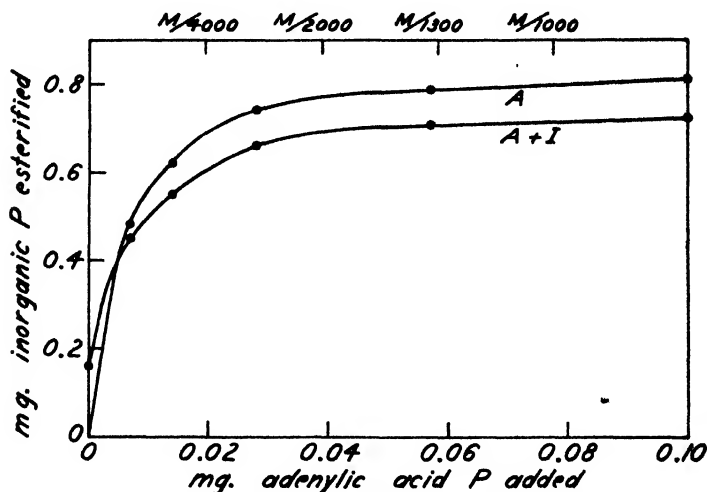


FIG. 2. Comparison of the effects of adenylic acid and of adenylic plus inosinic acids. An electro dialyzed extract was used. The experimental conditions were the same as in Fig. 1. Curve A shows the effect of adenylic acid alone, Curve A + I the effect of adenylic acid in the presence of a constant amount of inosinic acid ( $M/1500$ ). Since the addition of inosinic acid alone caused the esterification of 0.16 mg. of inorganic P, Curve A + I intersects the ordinate at this point and not at the origin.

Fig. 1,  $M/400$  inosinic acid gave about the same esterification as  $M/15,000$  adenylic acid.

It seems from these experiments that both adenylic and inosinic acid can act with the phosphorylizing enzyme, and that the enzyme has much more activity in combination with the former than with the latter nucleotide. In view of this result it was of interest to study the combined action of these nucleotides. It may be seen in Fig. 2 that adenylic acid produces less esterifi-

cation when it is acting in the presence of  $M/1500$  inosinic acid (and in another experiment in the presence of  $M/3000$  inosinic acid) than when it is acting alone.<sup>4</sup> Presumably part of the enzyme, by being in combination with inosinic acid, is not free to react with adenylic acid.

Curves ATP-13 and ATP-15 in Fig. 1 show that  $M/1000$  adenosinetriphosphate has hardly any effect in extracts practically free of pyrophosphatase. Concentrations several times higher than those shown in Fig. 1 have also been tested with Extracts 13 and 15, but no additional esterification of inorganic phosphate was observed. Entirely different results are obtained when the extracts contain pyrophosphatase which converts the added adenosinetriphosphate to adenylic acid; it may be seen in Fig. 1 that with Extract 14 adenosinetriphosphate displayed an activity similar to that of adenylic acid.<sup>5</sup>

The experiments indicate that adenosinetriphosphate cannot replace adenylic acid and act as coenzyme in the disruptive phosphorylation of glycogen with inorganic phosphate. The extracts did not form phosphocreatine from added creatine and adenosinetriphosphate. The analogous reaction between glycogen and adenosinetriphosphate obviously did not occur because the adenylic acid thereby formed would have imparted activity to the extracts.<sup>6</sup>

#### DISCUSSION

Of the three nucleotides investigated in the present paper adenylic acid displayed the greatest activity. In concentrations as low as  $M/30,000$ , it enabled completely inactive muscle extracts to esterify inorganic phosphate. Inosinic acid in concentrations of  $M/1000$  was only weakly active and adenosinetriphosphate was

<sup>4</sup> Such a result would not have been obtained if the action of inosinic acid depended on some mechanism for its reamination or if it contained adenylic acid as an impurity.

<sup>5</sup> The finding of Kendal and Stickland (9) that small concentrations of adenosinetriphosphate lead to the formation of glucose-1-phosphoric acid is probably due to the presence of pyrophosphatase in their muscle extracts.

<sup>6</sup> Ostern *et al.* (10) concluded that adenosinetriphosphate does not react with glycogen, while Lehmann and Needham (11) assume that such a reaction occurs.

practically inactive when added to muscle extracts free of pyrophosphatase.

In reactions previously described (12) in which adenylic acid acted as coenzyme, it has been shown to act as phosphate acceptor (for phosphate in organic combination, such as phosphocreatine or phosphopyruvic acid), thereby becoming adenosinetriphosphate, which then acts as phosphate donor (to hexosemonophosphate or creatine); in such reactions adenylic acid can be replaced by adenosinetriphosphate. However, in the reaction between glycogen and inorganic phosphate adenylic acid cannot be replaced by adenosinetriphosphate, showing that the transfer of inorganic phosphate involves a different mechanism. It should be mentioned, however, that adenosinediphosphate as a possible intermediate in this reaction has not yet been investigated.<sup>7</sup>

It has been shown previously (14) that the reaction which is catalyzed by minute amounts of adenylic acid in muscle extract—the synthesis of hexosemonophosphate from glycogen and inorganic phosphate—can be demonstrated in intact muscle. Adenylic acid is formed in muscle from adenosinetriphosphate when the latter transfers its mobile phosphate groups to hexosemonophosphate (10), a reaction which is now regarded as an obligatory step on the path from glycogen to lactic acid. The monophosphate is thereby converted to diphosphate, while part of the adenylic acid, by reacting with the phosphorylizing enzyme, will lead to the formation of new monophosphate. The fact that the concentration of monophosphate increases during short tetanic stimulation (15) indicates that it is resynthesized more rapidly than it accepts phosphate from adenosinetriphosphate. One may regard hexose-6-monophosphate, which is the only intermediate between glycogen and lactic acid found in unpoisoned muscle, as the immediate source of lactic acid and its reaction with adenosinetriphosphate as the limiting reaction for the rate of lactic acid formation in intact

<sup>7</sup> Since the above was written, adenosinediphosphate (Lohmann (13)) has been tested. It was found to give a typical coenzyme curve similar to that of Curve A-15 in Fig. 1. However, when incubated with glycogen without addition of inorganic phosphate, it did not give off its mobile phosphate group to form hexosemonophosphate. It seems therefore that the transfer of inorganic phosphate to glycogen does not involve the reaction  $H_2PO_4 + \text{adenylic acid} \rightleftharpoons \text{adenosinediphosphate}$

muscle. It seems significant, therefore, that the same reaction which causes the disappearance of monophosphate leads to the formation of adenylic acid which activates the synthesis of new monophosphate.

Adenylic acid reacts with phosphocreatine with the formation of adenosinetriphosphate and creatine (16). The reversible nature of this reaction makes it unlikely that it will go to completion in either direction under the conditions which exist in intact muscle. Adenylic acid also accepts phosphate groups from phosphopyruvic acid once the latter has been formed from hexosediphosphate.

A further process by which adenylic acid is known to be removed is its deamination to inosinic acid. It may be pointed out in regard to deaminase, pyrophosphatase, and other muscle enzymes, that while they are free to act in an uncontrolled fashion in muscle extracts, there undoubtedly exist mechanisms in intact muscle which regulate the rate as well as the sequence of their activity. It is unknown how rapidly adenylic acid is deaminated in intact muscle. Compared to adenylic acid rather high concentrations of inosinic acid are needed and even so it does not approach the activity of adenylic acid. An increase in the concentration of inosinic acid to  $m/1000$  would be accompanied by a decrease in the easily hydrolyzable ("pyrophosphate") P content of muscle of 6.2 mg. per cent, the latter being a change which could easily be detected. Changes of this magnitude have not been observed during ordinary activity of muscle, but they occur during extreme fatigue.

#### SUMMARY

1. The effect of nucleotides on the formation of hexosemonophosphate from glycogen and inorganic phosphate was investigated. Dialyzed rabbit muscle extracts were used which did not esterify inorganic phosphate without addition of nucleotides, which were free of deaminase and almost free of pyrophosphatase and which were unable to synthesize phosphocreatine.

2. In such extracts adenylic acid had a noticeable effect on the esterification of inorganic phosphate in concentrations of  $3 \times 10^{-5}$  molar and produced an effect approaching a maximum in  $10^{-3}$  molar concentrations. Compared to adenylic acid, inosinic acid displayed a weak activity even at  $10^{-3}$  molar concentrations, while adenosinetriphosphate was practically inactive at  $10^{-3}$  molar or higher concentrations.

3. When varying amounts of adenylic acid and a constant amount of inosinic acid ( $6 \times 10^{-4}$  molar) were added to an extract, to give 0.14 to 2 moles of adenylic acid per mole of inosinic acid, less esterification of inorganic phosphate was observed than when the same amounts of adenylic acid were added in the absence of inosinic acid.

4. Deaminase and pyrophosphatase were present in all freshly prepared extracts. These enzymes usually disappeared after the extracts had stood for 3 weeks in the refrigerator, at which time the phosphorylizing enzyme was still active, or they could be removed from freshly prepared extracts by adsorbing them on charcoal.

5. Adenosinetriphosphate, when added to muscle extracts containing pyrophosphatase, was about as active as adenylic acid to which it was converted in such extracts.

6. The rôle of nucleotides in intact muscle is discussed.

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## ELECTROKINETIC ASPECTS OF SURFACE CHEMISTRY

### V. ELECTRIC MOBILITY AND TITRATION CURVES OF PROTEINS AND THEIR RELATIONSHIP TO THE CALCULATION OF RADIUS AND MOLECULAR WEIGHT

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In solutions of proteins, the individual protein molecules<sup>1</sup> have, over a time average, a certain number of hydrogen ions bound,  $\epsilon$ . If certain assumptions are made (1), the titration curve may be used to obtain this value of  $\epsilon$  (or  $Q$  in electrostatic units)<sup>2</sup> by thermodynamic methods. A protein molecule, acted upon by an applied, homogeneous electric field, attains a mobility  $v$ , which is proportional to the difference in potential  $\zeta$  between the surface of the charged particle and an equal and oppositely charged layer situated statistically at a certain distance outward in the medium. These quantities,  $\zeta$  and  $v$ , are related by means of the equation of Helmholtz and Smoluchowski (2),

$$\zeta = \frac{C\pi\eta}{D} v \quad (1)$$

for unit field strength ( $C$  = a constant,  $\eta$  = the coefficient of viscosity, and  $D$  = dielectric constant).

\* John D. Jones Scholar, Biological Laboratory, Cold Spring Harbor, Summer, 1937.

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<sup>1</sup> The word molecule is used to designate a protein particle having a mean kinetic energy equal to  $3RT/2N$ .

<sup>2</sup>  $Q$ , the charge per molecule, is obtained by multiplying  $\epsilon$  by  $4.77 \times 10^{-10}$  electrostatic units, the electronic charge.

In addition, the theories of Gouy (3) and of Debye and Hückel (4) yield the expression relating  $\zeta$  to  $Q$ , the charge per particle,

$$Q = D\zeta r(\kappa r + 1) \quad (2)$$

where

$$\kappa = \sqrt{\frac{4\pi e^2}{DkT}} \sqrt{\sum_1^s n_i z_i^2} \quad (3)$$

$r$  = radius of the particle,  $k$  = Boltzmann's constant,  $e$  = electronic charge,  $T$  = absolute temperature,  $n_i$  = number of ions of the type  $i$  with valence  $z$  summated from 1 to  $s$ . Equation 2 may be strictly applied when  $\zeta e/kT \ll 1$ , a condition usually met near the isoelectric point in the case of protein solutions. The derivation and restrictions of these equations have been discussed at length elsewhere ((5) p. 100 ff.).

Equation 2 makes it possible to estimate the net charge of a protein molecule from electrokinetic data. Abramson ((1), (5) p. 151 ff.) after comparison of the thermodynamic and conductance theories, concluded that "in solutions of the same ionic strength, the electric mobility of the same protein at different hydrogen ion activities should be directly proportional to the number of hydrogen (hydroxyl) ions bound by each molecule" (1). This rule was confirmed by the available data on serum albumin, egg albumin, and normal and deaminized gelatin, and casein. Furthermore, Daniel (6) has shown that the agreement in curve shape exists under conditions of varied dielectric constant (in alcohol-water mixtures) as demanded by the theory.

Although direct proportionality could be demonstrated between electric mobility and titration curves, the values of  $Q$  calculated by the two methods were only in fair agreement. Moyer and Abels (7) have recently shown that much better agreement is secured between values of  $Q$  calculated for egg albumin from electrokinetic and titration data if Equation 2 is modified by changing the constant from 1 to 2. That is,  $(\kappa r + 1)$  is empirically replaced by  $(\kappa r + 2)$ , so that

$$Q = D\zeta r(\kappa r + 2) \quad (4)$$

This transformation was originally obtained (1) by the substitution of the expression for the diffuse double layer into that for

the rigid double layer but is at present without theoretical justification. However, by the use of this equation the electric mobilities of both egg albumin and R-phycoerythrin at one ionic strength were successfully calculated from data at another ionic strength (7). In view of the foregoing, it seems desirable to employ Equation 4 in further analyses of protein systems.

It is of considerable importance to have data at a constant ionic strength for the comparison of the serum proteins. In an earlier paper (8), comparison was made between the electrophoretic mobilities of horse serum albumin and pseudoglobulin in the dissolved state (9) and adsorbed on quartz or collodion particles. Complete agreement was secured, thus confirming (a) the accuracy of the microscopic method of electrophoresis and (b) the conclusions of Abramson (1) as to the negligible effect of the adsorption process on the ionization of certain proteins (see, however, the effects of adsorption on egg albumin as discussed by one of us (10)).

In this communication, close agreement is shown with the simple rule between titration curves and electric mobility data for the serum proteins over a wide range of pH. The experimental validity of Equation 4 is confirmed for these systems. In addition, methods are presented for the calculation of equivalent radii and molecular weights of proteins from electric mobility and titration curves.

### *Methods*

The horse serum albumin and pseudoglobulin were the same preparations as those used before (7). Measurements were made within 2 weeks after preparation. A portion of the globulins before separation of the pseudoglobulin and euglobulin by dialysis was dialyzed in 1 per cent NaCl until sulfate-free. This will be designated as the total globulins. Electrophoretic measurements were performed with the same horizontal microelectrophoresis instrument as that used in the previous papers of this series (7, 8, 10, 11) by techniques described in detail elsewhere (12). All measurements have been corrected to 25°. The method of preparing collodion suspensions and buffers has been described before (10). The titration curve of pseudoglobulin was determined by continuous titration with HCl and NaOH (quinhydrone electrode) under nitrogen. Calculations of acid- (base-) binding were made as before (7).

## EXPERIMENTAL

In Fig. 1 are shown the electric mobility-pH curves of collodion particles coated with horse serum albumin, pseudoglobulin (8, 9), and the total globulins at an ionic strength of 0.1. Attention is called to the agreement between the data for dissolved and adsorbed pseudoglobulin. Tiselius (9) has also presented data for the electric mobility of the dissolved total globulins (not

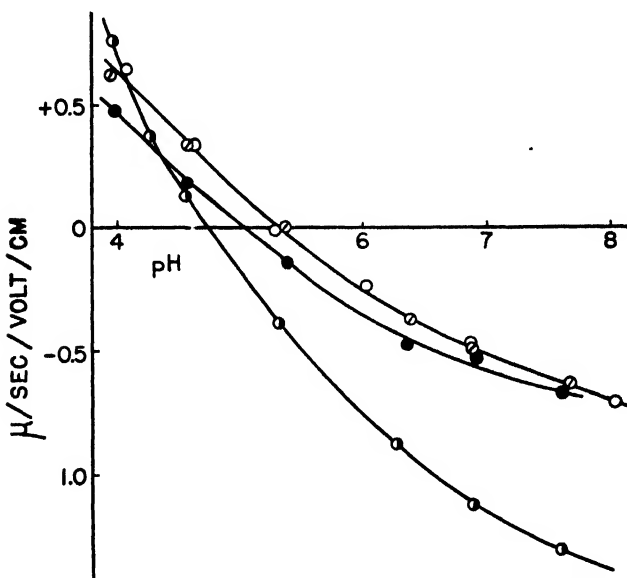


FIG. 1. Electric mobility curves of serum albumin (●), total globulins (●), and pseudoglobulin (dissolved, ○ (9); adsorbed, ⊙ (8)) in phosphate and acetate buffers at constant ionic strength (0.1). The smooth curves have been drawn free-hand to fit the points.

plotted here, but similar in shape to the pseudoglobulin curve). It is interesting to notice that our data for this inhomogeneous complex do not agree with his curve, presumably because a constituent of the total globulins, possibly a part of the fraction forming euglobulin on dialysis, is being preferentially adsorbed. Our curve is isoelectric at pH 5.0. Tiselius' curve is isoelectric at pH 5.2, nearer the isoelectric point of pseudoglobulin.

To test Abramson's rule, the serum albumin and pseudoglobulin

titration curves were plotted to fit the points of the mobility data (Fig. 2). Inasmuch as the molecules of serum albumin and pseudoglobulin are of different size, the scales for the two titration curves are different. Our pseudoglobulin titration curve was fitted to the mobility data by drawing separate smooth mobility and titration curves from the experimental results. At intervals along the pH scale, corresponding values were read off each and

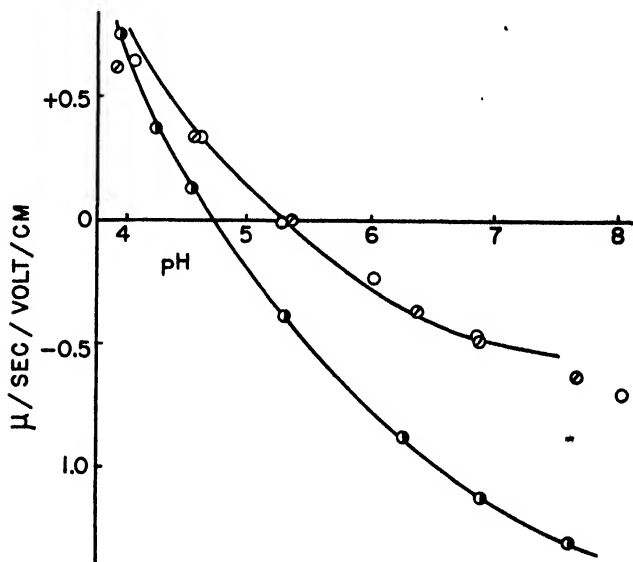


FIG. 2. Electric mobility curves of serum albumin (●) and pseudoglobulin (dissolved, ○; adsorbed, ⊙) in phosphate and acetate buffers at  $\mu_s = 0.1$ . The smooth curves are the titration curves for these proteins drawn to fit the mobility data as described in the text. This shows that over a wide range of pH the electric mobility of these proteins is proportional to their combining power with hydrogen and hydroxyl ions.

$v$  in  $\mu$  per second per volt per cm. was divided by  $h$ , the number of moles of acid (base) bound per gm. of protein. Except at pH values very close to the isoelectric point, where measurements are uncertain, the ratio  $v/h$  was found to be a constant so that  $v = 5000 h$ . The individual points on the titration curve were multiplied by this constant and plotted as a smooth curve for comparison with the electric mobility data. A titration curve for horse

serum albumin (13) was likewise fitted in this way; in this case, the constant of proportionality was 3300. At this ionic strength (0.1) serum albumin is isoelectric at pH 4.75.

The excellent agreement between the titration and mobility data for serum albumin over the range pH 4 to 8 is a striking confirmation of the proportionality existing between the two sets of results. The agreement is less complete in the case of pseudoglobulin, particularly above pH 7 where the quinhydrone electrode becomes less accurate, but the coincidence is remarkably close.

If Equation 1 is combined with Equation 2 or 4 there results, after division by  $e$ ,

$$\epsilon = \frac{C\pi\eta rv}{e} (kr + a) \quad (5)$$

$a$  may be 1 or 2, depending upon whether the theoretical Equation 2 or the quasiempirical Equation 4 is used; all quantities are in c.g.s. and E.S.U.

Comparison of this expression with the equation for calculating the charge from titration data,

$$\epsilon = hM \quad (6)$$

where  $M$  is the molecular weight of the protein, shows that if  $kr$  be kept constant,  $h$  should be directly proportional to  $v$ . At a constant value of  $DT$ ,  $\kappa^2$  is directly proportional to the ionic strength,  $\mu_i = \frac{1}{2} \sum_1^s n_i z_i^2$ , so that Abramson's rule should obtain.

In applying these equations to protein systems, Abramson made the following assumptions.

"(1) Complete dissociation of the protein salts or a constant fraction dissociated at different hydrogen ion activities. (2) The hydrogen ions bound act as if they were at or very close to the surface and uniformly distributed. (3)  $\eta$  and  $r$  do not change with pH. (4) The reaction of the protein with ions other than the  $H^+(OH^-)$  ion is negligible. (5) Only uni-univalent electrolytes are considered. (6)  $D$  and  $\eta$  of the medium can be used for their unknown values in the double layer, the effect of salts on  $D$  being unconsidered. (7) The distortion of the ion atmosphere remains essentially constant" ((5) p. 154).

The satisfactory agreement (7) between values of  $\epsilon$  calculated from Equations 5 and 6 is strong evidence that these assumptions

are justified. In the present case, the titration curves are not at constant ionic strength, yet proportionality still exists. Inspection of Fig. 1 in the paper of Moyer and Abels (7) shows that the titration curves measured in the presence or absence of added salt have nearly the same form. A shift in isoelectric point, similar to an adsorption of a practically constant amount of salt at all pH values, is occasioned by the salt. But when both curves are adjusted to the same isoelectric point the salt is seen to have produced a slight rotation without much change in shape. This was to have been expected from the work of Sørensen, Linderstrøm-Lang, and Lund (14) who postulated a family of such curves rotating about the isoelectric point.

Objection may be raised to the use of the electric mobilities of adsorbed serum albumin at  $\mu_i = 0.1$  in this comparison. This is felt to be valid, inasmuch as the electric mobilities of this protein adsorbed on quartz and collodion coincide exactly (1, 8) with data for dissolved serum albumin at  $\mu_i = 0.02$  (Fig. 3).

#### DISCUSSION

*Calculation of an Equivalent Radius*—Equation 5 offers a means of calculating the radius of a protein molecule if we make the additional assumptions that  $\epsilon$  and  $r$  are nearly independent of the ionic strengths under consideration and any shift in isoelectric point caused by a change in the ionic strength corresponds to the loss or gain of a constant charge over a pH range near the isoelectric point (where  $r$  is presumably independent of pH). Since at any given pH a certain number of hydrogen ions are attached to the protein molecule over a time average, the charge is mainly affected by pH rather than by changes in salt concentration at low ionic strengths. This is tantamount to assuming that, in addition to decreasing the thickness of the double layer, addition of salt involves the adsorption of a certain number of ions whose attachment is fairly independent of pH. The shift in isoelectric point on the addition of salts has been discussed by Abramson (15) and by Smith (16).

A value for  $C$ , the constant of proportionality in Equation 1, must be decided upon before Equation 5 can be employed. It was found by Hückel (17) that this constant should be 6 for a sphere. This was confirmed by Henry (18) for a small insulating



sphere but for large insulating particles the value 4 was deduced. For the intermediate size range, Henry has evolved two series expansions whose derivation rests upon the assumption of the validity of the Debye-Hückel approximation (Equation 2). For similar values of  $\kappa r$ ,  $C$  is only slightly different. Abramson (15) and Moyer and Abels (7) have formed the proportion (from Equation 5)

$$\frac{v_1}{v_2} = \frac{\kappa_2 r + a}{\kappa_1 r + a} = b \quad (7)$$

by assuming that cancellation of the constant of proportionality is valid for the range of  $\kappa r$  investigated here. Solving for  $r$ , we obtain

$$r = \frac{a(1 - b)}{b\kappa_1 - \kappa_2} \quad (8)$$

an expression by which the radius may be calculated if electric mobility-pH curves are determined at two ionic strengths (corresponding to  $\kappa_1$  and  $\kappa_2$ ).

In practise, we have found it best to determine the constant  $b$  for a series of ratios,  $v_1/v_2$ , over a range of pH near the isoelectric point. When the isoelectric point is shifted by increasing ionic strength, it has been our practise to plot both curves and determine graphically the value of the constant  $k$  needed to be added algebraically to bring both curves to the same isoelectric point. This is introduced into the ratio as  $v_1/(v_2 + k) = b$ .

Ultracentrifugal measurements of Svedberg and Sjögren (19) have shown that the molecules of horse serum albumin have an asymmetry number of 1.29; *i.e.*, they are not spherical. In cases in which the protein molecule departs slightly from sphericity, an "equivalent radius" should be obtained. This ought not to agree with the radius from density measurements. In Fig. 3 are shown the available electric mobility data for serum albumin at two ionic strengths. Upon adjustment of the isoelectric points to a common value at pH 4.75,  $b$  was found to be quite constant. The value for the radius as calculated from Equation 8 is presented in Table I. Values for the other proteins were computed from data of Tiselius (9) for the electric mobility of egg albumin and R-phycoerythrin in sodium and barium acetate buffers. These data have been discussed before (7). The phycoerythrin calculations

were based on a smooth curve drawn through Tiselius' points; the smooth curves in Fig. 4 of the paper by Moyer and Abels (7) were used for egg albumin. Values for the radii calculated from Stokes' law, Einstein's diffusion equation and densities (20) are

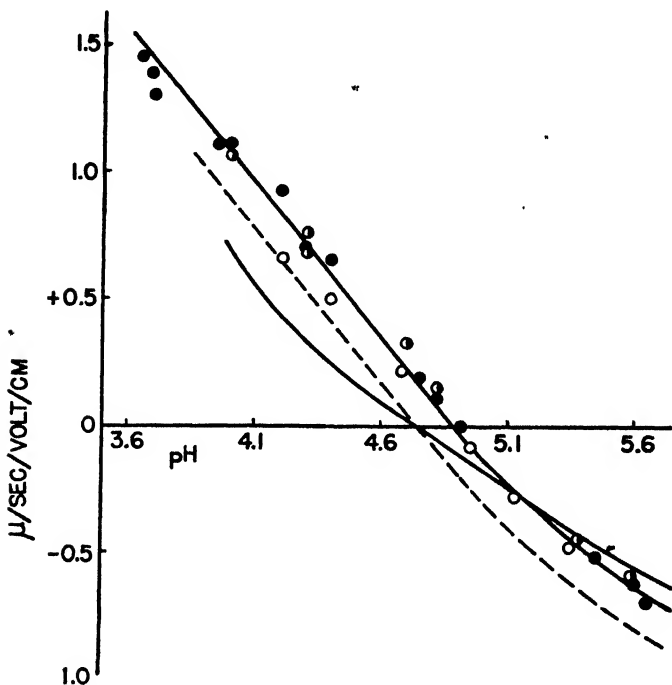


FIG. 3. Electric mobility data for serum albumin in 0.02 *M* acetate buffers. ○, data of Tiselius for dissolved serum albumin; ●, data of Abramson for serum albumin adsorbed on quartz particles; ◐, data of Moyer for this protein adsorbed on collodion particles. The smooth curve with its isoelectric point at pH 4.90 was chosen as the best free-hand curve to fit the data. For comparison, the smooth curve for this protein at  $\mu_i = 0.1$  has also been drawn (isoelectric point at pH 4.75). The dashed curve depicts the shape of the mobility curve at  $\mu_i = 0.02$  after the isoelectric point is adjusted to pH 4.75.

presented in Table I for comparison. In a recent paper, Svedberg (21) has presented values for the molecular weights and diffusion constants,  $D_{20}$ , based on unpublished data, which differ from his earlier results. Both sets of data have been used for calculation,

the more recent data being presented second. Notice in Table I that the empirical value of the constant  $a$  ( $a = 2$ ) gives in each case values for  $r$  which agree much more closely with other methods. Attention is called to the excellent agreement between our value of 3.40 for the equivalent radius of serum albumin and that calculated from diffusion. For this aspherical protein, neither Stokes' law nor density values give results in agreement with ours. It is possible that effects of orientation, not encountered in diffusion or electrophoresis, have caused the divergence. Equations

TABLE I

*Comparison of Radii of Protein Molecules Calculated from Electrokinetic Data with Values Determined by Other Methods*

Protein	Radius, in $m\mu$ , calculated from				
	Equation 8		Einstein's equation	Stokes' law	Density
	$a = 1$	$a = 2$			
Egg albumin (a)*	1.08	2.17	2.23	2.18	2.17
“ “ (b)			2.76	2.20	2.32
R-Phycocerythrin (a)†	2.60	5.20	4.09	3.93	3.94
“ (b)			5.35	4.03	4.41
Serum albumin‡	1.70	3.40	3.32	2.44	2.70

\* (a)  $M = 34,500$ ,  $D_{20} = 9.58 \times 10^{-7}$ ; (b)  $M = 42,200$ ,  $D_{20} = 7.76 \times 10^{-7}$ .

† (a)  $M = 208,000$ ,  $D_{20} = 5.22 \times 10^{-7}$ ; (b)  $M = 291,000$ ,  $D_{20} = 4.00 \times 10^{-7}$ .

‡  $M = 67,000$ ,  $D_{20} = 6.45 \times 10^{-7}$ .

tion 8 is highly sensitive to slight changes in  $b$ , so that the agreement between our values and those based on Svedberg's data is better than might have been expected and more significant, therefore, can be attached to the agreement.

*Estimation of Molecular Weights from Electric Mobility and Titration Curves*—Moyer and Abels (7) found that when  $a$  was made equal to 2 the charge calculated from the titration curve of egg albumin agreed with that calculated from its electric mobility curve. Combination of Equations 5 and 6 under these assumptions gave the expression

$$M = \frac{6\pi\eta r v_1}{h e} (\kappa_1 r + 2) \quad (9)$$

for unit field strength, with the constant  $C = 6$ . Introduction of the radius calculated in the preceding section and the corresponding values for  $h$ ,  $v_1$ , and  $\kappa_1$  permits the calculation of the molecular weight from electric mobility and titration data (Table II). Likewise, smooth curves drawn through the electric mobility data of Tiselius (9) (at  $\mu_i = 0.02$ ) and the titration curve of Moyer and Abels (7) for egg albumin at constant ionic strength have been used to calculate its molecular weight. The data in Fig. 3 have

TABLE II

*Comparison of Molecular Weights Calculated from Electrokinetic and from Ultracentrifugal Data*

Protein	Mol. wt. calculated from	
	Equation 9	Ultracentrifuge data
Egg albumin .....	37,000	34,500*
		42,200†
Serum albumin .....	66,600	67,000†

\* Svedberg (20).

† Svedberg (21).

been used for the serum albumin calculations.<sup>3</sup> There seem to be no titration data for R-phycoerythrin, so it could not be used. The agreement with ultracentrifugal data (Table II) is quite satisfactory. It should be emphasized that calculation of molecu-

<sup>3</sup> Serum albumin may be used as an example of the method of computation.  $r = 3.40 \times 10^{-7}$  cm.,  $\eta = 8.95 \times 10^{-3}$  poise,  $e = 4.77 \times 10^{-10}$  e.s.u.,  $\mu_i = 0.1$ . Reducing  $v$  to cm. per second, the proportionality between  $v$  and  $h$  becomes  $v = 0.330 h$ . This must be divided by  $1/300$  e.s.u. to convert volts to e.s.u. of potential. Inserting these values in Equation 9, we have

$$M = \frac{6 \times 3.14 \times 8.95 \times 10^{-3} \times 3.40 \times 10^{-7} \times 0.330}{3.33 \times 10^{-3} \times 4.77 \times 10^{-10}} (0.328 \times 10^8 \sqrt{0.1} \times 3.40 \times 10^{-7} + 2)$$

$$M = 6.66 \times 10^4$$

lar weights by this method needs only a titration curve and two mobility curves at different ionic strengths.<sup>4</sup> If values of  $C$  from Henry's equations had been used, the molecular weight of serum albumin would have been slightly lower. Table I has shown, however, that the simple Debye approximation upon which Henry's equations rest does not lead to correct values of  $r$  for proteins. Until this point is clarified, we have decided to use  $C = 6$ , which is, in fact, the value found by Hückel.

The excellent agreement between the values for the molecular weight of serum albumin indicates that the titration curves of this protein, at  $\mu_i = 0$  and  $\mu_i = 0.1$ , must lie very close together over the pH range used here. In other words,  $\epsilon$  here must be only slightly affected by ionic strength. Calculations with titration data at the same ionic strength as the electrophoresis measurements would have been preferable but no data were available.

#### SUMMARY

1. Electric mobility curves for collodion particles in solutions of horse serum albumin and total globulin are presented for comparison with earlier data on pseudoglobulin at the same ionic strength.

2. The electric mobilities of adsorbed and dissolved molecules of serum albumin and of pseudoglobulin had been found to be identical, within the limits of error (8). However, collodion particles with an adsorbed film of protein from the total globulins do not give the same electric mobility curve or isoelectric point as the dissolved total globulins. This is presumably due to selective adsorption of a component from the total globulin complex.

3. In solutions of the same ionic strength the electric mobilities of both serum albumin and pseudoglobulin were found to be proportional, over a wide range of pH, to the hydrogen ions bound.

4. It is shown that radii of protein molecules may be calculated from electric mobility data. Application of the equations to data for egg albumin, serum albumin, and R-phycoerythrin yielded values in close accord with the radii calculated by other methods.

5. By combining the thermodynamic and conductance methods for determining the net charge of proteins, an equation was derived

<sup>4</sup> It should be noticed that these calculations of  $M$  and  $r$  are all based on data limited to the pH-stability ranges (20, 21) of the proteins under consideration.

from which the molecular weight of a protein can be calculated from data on its combining power with acids and bases and its electrophoresis curves. The molecular weights of egg and serum albumin, thus estimated, were found to be in complete agreement with ultracentrifugal data.

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# THE QUANTITATIVE PRECIPITATION OF CITRIC ACID

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In an attempt (1) to obtain information on the nature of the unidentified constituents of urine from an analysis of its titration curve, it was found advantageous to remove citrate by some preliminary treatment. The precipitation of citric acid as the calcium salt from aqueous solution was found to be incomplete. Its precipitation from urine, however, was found to be almost complete. Further experimentation showed that this more complete precipitation from urine than from aqueous solution could be attributed to the phosphate present in the urine. Citrate, calcium, and phosphate apparently formed an insoluble complex.

This behavior was studied in greater detail because it promised to furnish a means for the complete removal of citrate from aqueous solution and also because it may be encountered at unexpected places in the determination of other organic acids. These studies have shown that the concentrations of calcium and of phosphate in the solution determine the amount of citric acid precipitated. When these salts are present in sufficient amounts and suitable proportions, citric acid is quantitatively precipitated.

## EXPERIMENTAL

Solutions containing varying amounts of calcium chloride, potassium acid phosphate, and citric acid were prepared in centrifuge tubes. Normal sodium hydroxide was added to each tube, slowly and with constant stirring, in slight excess of the amount required to make the solution blue to litmus. After standing for a short time the tubes were centrifuged and the clear supernatant liquid was decanted from the white gelatinous precipitate. Each precipitate was dissolved in 1.66 N sulfuric acid; the resulting



solutions were transferred to volumetric flasks of convenient size and brought to volume with the acid. The amount of citrate in these solutions was determined from the amount of  $\text{CO}_2$  liberated when they were oxidized with  $\text{KMnO}_4$  in the Van Slyke manometric apparatus (2).

A number of these determinations are recorded in Table I. The first two demonstrate the extreme sensitivity of the reaction. In the presence of only small amounts of phosphate and calcium,

TABLE I  
*Influence of Phosphate and Calcium on Completeness of Citrate Precipitation*

Experiment No.	Constituents of precipitating solutions*				Citrate precipitated per cent
	Citric acid		$\text{CaCl}_2$	$\text{KH}_2\text{PO}_4$	
	mg.	m.-eq.	m.-eq.	m.-eq.	
1	0.035	0.0054	1.10	0.21	80
2	0.13	0.0203	8.2	1.38	95
3	11.8	0.184	10.9	0.00	2
4	11.8	0.184	10.9	0.69	78
5	11.8	0.184	10.9	1.38	94
6	11.8	0.184	10.9	2.76	98
7	11.8	0.184	10.9	5.49	99
8	11.8	0.184	10.9	7.22	99
9	11.8	0.184	10.9	10.98	19
10	11.8	0.184	10.9	13.74	8
11	11.8	0.184	21.8	10.98	100
12	27.4	0.428	27.4	8.82	101
13	82.2	1.28	27.4	8.82	99
14	109.5	1.71	27.4	8.82	97
15	137.0	2.14	27.4	8.82	94

\* In each experiment the indicated amount of citric acid was contained in 100 cc. of solution. The addition of calcium chloride, potassium phosphate, and sodium hydroxide increased this volume from 2 to 20 per cent. These changes in volume were insufficient to affect the amount of citrate precipitated significantly.

80 per cent of the citric acid in a 0.035 mg. per cent solution was precipitated. The use of larger amounts of calcium and phosphorus might have been accompanied by more complete precipitation, but this could not be shown experimentally because of the limits of the analytical procedure. Practically complete precipitation was obtained from a solution containing 0.13 mg. of citric acid per 100 cc. with the amounts of calcium and phosphate indicated.

The remaining experiments demonstrate more clearly the part phosphate plays in this precipitation of citrate. When no phosphate was present, practically no citrate was precipitated. Increased amounts of phosphate resulted in the precipitation of increased amounts of citrate, until complete precipitation occurred. Beyond this, citrate precipitation again became incomplete. This incomplete precipitation occurred whenever (Experiments 9 and 10) the amount of calcium in the mixture was not sufficient to unite with all the phosphate and citrate. Use of more calcium (Experiment 11) again produced complete precipitation. In the last four experiments, 2.94 mm of phosphate were sufficient to precipitate quantitatively about 100 mg. of citric acid.

#### DISCUSSION

It would probably be difficult to obtain significant information on the structure of the precipitate formed in these experiments. It might be expected to contain some calcium phosphate; the variable composition of this compound (3) would complicate the interpretation of analyses for the mole ratios of phosphate, calcium, and citrate in the precipitate. The fact that citrate is precipitated only when calcium ion is present in excess of the amount necessary to react with phosphate and citrate seems to indicate that it forms an insoluble calcium complex which is readily dissociated. The precipitation of only small amounts of citrate under certain conditions in which large amounts of calcium phosphate are precipitated (Experiments 9 and 10) indicates that citrate precipitation is not due to adsorption on calcium phosphate.

#### SUMMARY

When a solution of citric acid, containing suitable amounts of calcium and phosphate, is made alkaline, the citric acid is quantitatively precipitated. Calcium must be present in excess of the amount necessary to react with phosphate and citrate. Increasing amounts of phosphate are required to precipitate larger amounts of citrate.

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## THE BUFFER ACTION OF UNIDENTIFIED URINE CONSTITUENTS

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In 1922, Van Slyke (1) developed simple methods for determining the buffer values of solutions. Equations were derived by which these values may be calculated for solutions of known composition. When the composition is not known, buffer values may be experimentally determined from the slope of the titration curve. At any pH, the total buffer value of a solution is the sum of the buffer values of the individual constituents of that solution.

The use of these principles offers an opportunity for obtaining information concerning the nature of unknown acidic or basic constituents of a solution. Normal urine may contain a number of these unknown constituents. According to Van Slyke and Palmer (2) the total organic acid present in urine, determined by titration after the removal of phosphate and carbonate, is from 3 to 5 times the amount of creatinine present (expressed as cc. of 0.1 N solution). On this basis, a normal representative urine contains from 10 to 300 cc. more 0.1 N acid than the sum of the known constituents titrated in this range.<sup>1</sup> The true amount of organic acid unaccounted for may be somewhat higher than this value because of the partial removal of both uric (4) and citric (5) acids in the process of removing carbonate and phosphate, preliminary to the organic acid titration.

This estimate of unidentified urinary constituents includes only those substances which dissociate below pH 8.0. There is

<sup>1</sup> These calculations are based on the representative urine sample given by Bodansky (3), an assumed citric acid excretion of 0.8 gm., and the dissociation constants used by Van Slyke and Palmer (2).

no information available on the total amount of excreted acid or base which dissociates above this pH.

This paper presents a method for determining the pH ranges over which these unidentified constituents exert buffer action. Urine was first treated to remove citrate, phosphate, and carbonate, as will be described later. The total buffer action of this treated urine sample was then determined at appropriate intervals from the slope of an electrometric titration curve of the urine. That part of the total buffer action attributable to hydrogen and hydroxyl ions was determined from the titration of an aqueous solution of hydrochloric acid. That part of the buffer action attributable to known acidic and basic constituents was calculated from their respective dissociation constants and analytical data. The remaining buffer action is attributable to undetermined constituents. Since nearly all organic compounds which may be excreted into the urine dissociate to some extent within the pH limits of this titration, this sort of experimental approach may prove to be a useful tool in detecting the excretion of some unsuspected substances. It may also aid in their identification by indicating their dissociation constants.

#### EXPERIMENTAL

##### *Preliminary Treatment of Urine*

To a volume of urine equivalent to a 3 hour sample, 33 cc. of 50 per cent  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  solution were added. The solution was then made acid to Congo red paper (less than pH 3) with hydrochloric acid, diluted to a volume of 600 cc., aerated for at least 15 minutes to remove carbon dioxide, and allowed to stand overnight in the refrigerator.

On the following day the urine was filtered, and 4 cc. of a 10 per cent  $\text{KH}_2\text{PO}_4$  solution were added for each 100 cc. of filtrate. The urine was then heated to  $50^\circ$  and, with vigorous mechanical stirring, 10 per cent  $\text{NaOH}$  was added a few cc. at a time from a burette until the solution became alkaline to litmus; then an excess of 1.5 cc. of the alkali was added for each 100 cc. of original sample. The dilution produced by these reagents was brought up to 12 cc. per 100 cc. of urine by the addition of water. After the solution was allowed to stand for a half hour, it was filtered

and the filtrate made acid to litmus and preserved with toluene for analysis and electrometric titration.

This preliminary procedure was designed to remove those substances which interfere in electrometric titration. Phosphate was removed as precipitated calcium phosphate. Preliminary acidification changed the carbonate in the urine to carbonic acid and caused excess uric acid to precipitate. The carbonic acid was removed by aeration and the precipitated uric acid by filtration. Both of these substances would have interfered in electrometric titration, the uric acid by dissolving during the course of titration and the carbonic acid by being liberated as carbon dioxide when hydrogen was passed through the solution. Additional phosphate and the large excess of calcium were added to the original urine samples because these substances facilitated the removal of citrate from solution. Citric acid is precipitated with calcium and phosphate when the solution is made alkaline (6). The phosphate added in the preliminary urine treatment was sufficient to effect the removal of citrate when present up to 4.8 gm. per day's sample. This corresponds to the fastest rate at which citrate has been reported to be excreted into urine (7). A small amount of citrate in excess of the maximum recorded would be precipitated by phosphate present in normal urine.

The complete removal of citrate is to be preferred to inclusion of citrate in the general procedure of analysis and calculation of buffer action used in the case of the various other urinary constituents. If additional calcium were added to remove only the phosphate present in the urine, some of the citrate would also be removed, leaving a relatively small but variable amount in the urine. The methods available for the determination of these small amounts of citrate are somewhat involved. Furthermore, the calculation of the buffer action of this citrate would be complicated by the fact that the titration curve of citric acid is influenced by the presence of the varying amount of calcium present in the solution (8). The addition of a large excess of calcium for the complete removal of citrate is of further advantage in that it increases the ionic strength of the titrated solution to such an extent that variations in the ionic strength attributable to varying amounts of phosphate and carbonate in the original urine would be relatively small.

The removal of citrate, phosphate, and carbonate before titration is also of advantage because it diminishes the error in the determination of the buffer value of unidentified urine constituents. This last value is determined as the difference between the values calculated for the analytically determined constituents and the total buffer action. Errors in determination of known substances present in large amount or in the determination of total buffer action would be reflected as proportionately larger errors in the buffer action of unidentified urine constituents. Precision is increased by preliminary removal of as many titratable substances as possible.<sup>2</sup>

#### *Determination of Buffer Values*

Titration was performed on 90 cc. samples of previously treated urine made acid to Congo red with 10 per cent HCl in a 150 cc. flask held in a constant temperature water bath at 30°. In the stopper to the flask were inserted an agar bridge, a small stirring device, a burette for the addition of carbonate-free alkali (0.115 N), two small hydrogen electrodes of the Hildebrand type (prepared as suggested by Britton (10)), and a tube for the escape of hydrogen. A calomel cell, specially designed for partial immersion in the constant temperature bath, was connected to the reaction mixture by means of the agar bridge. In order to facilitate precise addition of alkali, the burette was drawn to a small point and extended into the titration mixture. Potential readings were made to 0.1 millivolt at about 10 millivolt intervals and the titrations duplicated. The electrodes were slowly poisoned; usually the potential was found to lag about 2 millivolts after 30 minutes use, when checked against and replaced by a freshly platinized electrode. 0.05 M potassium acid phthalate was used as a standardizing solution.

The ammonia content of the urine samples was found to diminish about 3 per cent during the titration period. This was de-

<sup>2</sup> The results of titration of untreated urine samples have been reported by other workers (9). The precipitation of calcium phosphate during titration, the large corrections made necessary by the presence of carbonate and phosphate, and the uncertain reliability of quinhydrone electrode measurements of urine samples in the pH range in which it was used lead one to doubt the significance of the measurements.

terminated from the amount aerated over by the hydrogen, and from analysis of urine samples before and after the titrations. This loss was not sufficient to affect significantly the titration curve.

The experimental buffer values were obtained directly from the slope of the titration curves, as the amount of 0.115 N alkali required to cause a change of 50 millivolts. The buffer values for the analyzed constituents were calculated by the use of the equation given by Van Slyke (1), modified for the units used in reading the buffer value from the titration curve.<sup>3</sup> The buffer action of hydrogen and hydroxyl ions was determined by titration of solutions which contained about 8 cc. of 0.1 N HCl, 5 cc. of 25 per cent  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 5 cc. of 15 per cent NaCl, and sufficient water to make the usual titration volume. These later values vary directly with the volume of the titrated solution. Corrections were therefore applied for the difference in volume between these titrations of hydrochloric acid and the titrations of the various urine solutions. The buffer value of other constituents is not affected by change in volume during titration. The experimentally determined buffer values of hydrogen and hydroxyl ions agreed closely, especially in the acid range, with values calculated from the theoretical equation (1).

The dissociation constants of the more important urine constituents, creatinine and ammonia, were determined from titration curves of these substances made at the same temperature

<sup>3</sup> According to Van Slyke

$$\beta = \frac{dB}{dpH} = 2.3 \frac{K' [H^+] C}{(K' + [H^+])^2}$$

$\beta$  = buffer value, expressed as equivalents of strong acid or base required to change the pH 1 unit;  $K'$  = dissociation constant;  $C$  = equivalent amount of substance. In order to express buffer value in terms of cc. of 0.115 N NaOH required for a 50 millivolt (0.832 pH) change

$$\beta' = \frac{dB}{dpH} \times \frac{1}{0.115} \times 0.832 \times 1000$$

In order to facilitate calculations, the values for an equivalent concentration given by this equation were plotted on a curve such as given by Henderson (11) and this used for each constituent substance by multiplying the number of equivalents by the values on this curve.



and similar salt concentration as were used for urine titrations. They agreed closely with published values for slightly different temperatures and salt concentration (9, 12). Other dissociation constants were taken directly from the literature.<sup>4</sup>

### *Methods*

Ammonia was determined by the aeration procedure ((15) p. 547), except that ammonia was aerated into boric instead of hydrochloric acid.<sup>5</sup> Control determinations gave theoretical recovery. Hippuric acid was determined by the method of Griffith (17), but when it became evident that by this method the small amount present in normal urine was not determined, calculations for the buffer action of hippuric acid were based on a daily excretion of 0.4 gm. Significantly larger amounts would have been noticed in the hippuric acid analyses. Determination of creatine and creatinine by the Folin colorimetric methods (18) showed that about 10 per cent of the total creatinine was present, in each case, as creatine. Buffer values, however, were based on the total creatinine figures and the dissociation constant of this substance. Uric acid determinations were made by the indirect method of Christman and Ravitch (19), modified for use with small amounts of material, and the results corrected for incomplete precipitation of uric acid. Only insignificant, normal amounts of lactic acid and acetone bodies were found by the methods of Friedemann, Cotonio, and Shaffer (20), and Van Slyke (21) respectively. Amino acids were determined by the manometric method of Van Slyke and Kirk ((15) p. 926). Urea was not determined experimentally on each sample, but when it became apparent that this substance would also exert buffer action over the extreme acid range, its buffer action was calculated from an excretion of 13 gm. per day, the amount excreted by the subject on a similar diet.

<sup>4</sup> Calculations were based on the following acid dissociation constants: creatinine =  $1.61 \times 10^{-8}$ , ammonium hydroxide =  $7.03 \times 10^{-10}$ , uric acid (13, 14) =  $2.0 \times 10^{-6}$ , and  $6.9 \times 10^{-10}$ , hippuric acid (12) =  $2.0 \times 10^{-4}$ , urea ((12), corrected for dissociation of water at 30°) =  $1 \times 10^0$ .

<sup>5</sup> These determinations were performed before the details of a similar procedure were reported by others (16).

*Results*

The data in Table I show the distribution of buffer action among the various constituents of a urine sample, previously treated to

TABLE I

*Distribution of Buffer Action among Constituents of Normal Urine Sample Previously Treated to Remove Phosphate, Citrate, and Carbonate*

Buffer units are given as cc. of 0.115 *N* NaOH required to cause a change of 0.832 pH unit (50 millivolts) in a solution which contained 46.8 mg. of creatinine.

pH	Total buffer	Urea and ammonia	Uric acid	Creatinine	Hippuric acid	H ions and OH ions	Unidentified constituents
2.29	10.6	0.52		0.03	0.03	7.70	2.3
2.63	5.82	0.23		0.06	0.06	3.55	1.91
2.96	3.85	0.11		0.10	0.13	1.70	1.81
3.29	2.95	0.05		0.19	0.19	0.88	1.64
3.63	2.43	0.02		0.39	0.22	0.41	1.39
3.96	2.45			0.76	0.19	0.21	1.29
4.29	2.44		0.03	1.24	0.13	0.10	0.94
4.63	2.50		0.05	1.65	0.07	0.06	0.67
4.96	2.26		0.09	1.65	0.03	0.03	0.46
5.29	1.81		0.15	1.24	0.02	0.02	0.38
5.63	1.25		0.18	0.76	0.01		0.30
5.96	0.83		0.17	0.39			0.27
6.29	0.55	0.02	0.12	0.19			0.22
6.62	0.50	0.04	0.07	0.10			0.29
6.95	0.40	0.09	0.04	0.03			0.24
7.29	0.50	0.17	0.02				0.31
7.62	0.75	0.36	0.02				0.37
7.95	1.29	0.69	0.04			0.01	0.55
8.29	2.22	1.33	0.07			0.03	0.79
8.62	3.35	2.19	0.12			0.06	0.98
8.95	4.70	2.99	0.17			0.11	1.43
9.29	5.00	3.08	0.18			0.20	1.54
9.62	4.30	2.37	0.15			0.35	1.43
9.95	3.45	1.49	0.09			0.62	1.25
10.28	3.10	0.80	0.05			1.15	1.10
10.45	3.15	0.58	0.04			1.75	0.78

remove phosphate, citrate, and carbonate. The buffer action due to unidentified urine constituents is the difference between the total, experimentally determined buffer action and the sum

of the buffer values of the individual constituents recorded in Table I.

From one-third to one-half of the unidentified buffer action at the more alkaline range is attributable to free amino acids (Van Slyke manometric determination). A definite maximum occurs at about the mean pH of their dissociation constants. Because no information is available on just what these amino acids may be, their buffer maxima could not be attributed to any definite pH value and the curve corrected for their presence. Other basic compounds measured in the formaldehyde titration and perhaps represented by those groups which react slowly with nitrous acid ((15) p. 926) may be largely responsible for the remainder of this buffer action. Phenolic compounds also are responsible for a part of the unidentified buffer action in this region. Assuming that all of a normal excretion of 0.5 gm. was excreted as phenol, they would exert a maximum of about 0.25 unit at about pH 10.

A part of the undetermined buffer action in the acid range may be attributed to the acid group of those amino compounds whose basic group is responsible for buffer action at higher pH values. The amount of buffer action attributable to these amino acids cannot be calculated, however, since neither the number nor the dissociation constants of the acid groups are known. Their maximum action would lie at about pH 2.5. A very small part may also be attributed to the small, normal amounts of lactic acid and acetone bodies which were found to be present. In addition, organic acid of unknown composition may account for a small part of the buffer action.

Between the pH limits of 5.5 and 7.5 the unidentified constituents show little, but a constant buffer action. The absence of a maximum within these limits indicates that this buffer action is produced by at least two different substances whose dissociation constants lie within this range.

In order to obtain information on what factors control the excretion of these unidentified urine constituents, samples were collected during the ingestion of normal diets and after the previous administration of sodium bicarbonate, ammonium chloride, citric acid, and sodium citrate (Table II). The buffer action of these samples attributable to unidentified constituents was de-

terminated in the same manner as before. In order to make the different experiments comparable, buffer values were calculated in each case on the basis of a urine sample which contained 45.2 mg. of creatinine (Table III).

The variations in the unidentified buffer action after the ingestion of acid and alkali are within the variations obtained with normal samples. This indicates that normal urine contains no quantitatively important constituents other than ammonium, citrate, and carbonate, which are excreted in varying amounts with change in urine acidity.

The experiments in which sodium citrate was ingested were performed with the purpose of identifying, from the dissociation

TABLE II  
*Collection of Experimental Urine Samples*

Substance administered	Time of collection	pH	Free amino N m.-eq.*
(a) Normal sample.....	10.50 p.m.- 8.00 a.m.		0.187
(b) " " .....	9.45 " - 7.00 p.m.	5.48	0.125
(c) 30 gm. NaHCO <sub>3</sub> .....	9.10 a.m.- 5.40 "	8.52	0.161
(d) 17 " " .....	9.50 " - 1.00 "	8.28	0.115
(e) 5 " NH <sub>4</sub> Cl.....	9.50 " -12.50 "	5.30	0.076
(f) 12 " citric acid + 17 gm. sodium citrate .....	10.30 p.m.- 7.30 a.m.		0.086
(g) 12 gm. sodium citrate.....	9.30 a.m.-12.45 p.m.	7.98	

\* Calculated for a volume of urine which contained 45.2 mg. of creatinine.

constant, excess organic acid which is said to be excreted after the ingestion of citrate. After administration of 35 gm. of citric acid to dogs, Sherman, Mendel, and Smith (5) found an increased organic acid excretion of about 280 cc. of 0.1 N acid not due to increased citrate excretion. Schuck (22) reported that in man the ingestion of 12 gm. of citric acid, taken in several doses, had little or no effect on organic acid excretion, but that the ingestion of an equivalent amount of sodium citrate caused a large increase of about 700 cc. of 0.1 N organic acid, not attributable to increased citric acid excretion. So large an amount of organic acid would cause a marked change in the curve of buffer action of unidentified constituents by which the dissociation constant of the acid could

be determined. The experiments shown in Tables II and III, however, show no significant increase in unidentified organic acids after the ingestion of sodium citrate or of a mixture of sodium citrate and citric acid.

TABLE III

*Variations in Buffer Action of Urine Due to Amino Acids and Other Unidentified Constituents*

Buffer units are given as cc. of 0.115 N NaOH required to cause a change of 0.832 pH unit in a solution which contained 0.404 milli-equivalent (45.2 mg.) of creatinine. This amount of creatinine would produce a maximum buffer effect, measured in the units here used, of 1.66.

pH	Urine samples collected after previous administration of						
	(a) Normal diet	(b) Normal diet	(c) Bicarbonate	(d) Bicarbonate	(e) Ammonium chloride	(f) Citrate	(g) Citrate
2.25	2.53		1.55				
2.50	2.79	1.96	2.05	2.90	1.75	2.43	2.35
3.00	2.49	1.66	1.85	2.82	1.68	2.47	1.80
3.50	2.40	1.52	1.78	2.75	1.47	2.38	1.55
4.00	1.90	1.16	1.29	2.50	1.06	1.74	1.28
4.50	1.03	0.70	0.84	1.95	0.85	1.18	0.88
5.00	0.77	0.50	0.66	1.39	0.59	0.71	0.70
5.50	0.45	0.32	0.44	0.62	0.50	0.54	0.47
6.00	0.36	0.27	0.22	0.22	0.34	0.34	0.37
6.50	0.37	0.28	0.21	0.22	0.24	0.27	0.25
7.00	0.43	0.24	0.30	0.29	0.22	0.26	0.26
7.50	0.53	0.33	0.37	0.56	0.44	0.42	0.39
8.00	0.94	0.66	0.58	1.10	0.60	0.65	0.73
8.50	1.60	0.95	0.93	1.53	0.97	1.02	1.10
9.00	2.01	1.46	1.19	1.78	1.45	1.23	1.38
9.50	1.59	1.47	1.18	1.71	1.26	1.13	1.19
10.00	1.24	1.27	0.96	1.30	0.87	0.75	0.96
10.50	0.85	0.88	0.78	0.90	0.69	0.50	0.45

It is difficult to explain this contradiction. The experiments of Schuck indicate that in man excess organic acid is excreted only when citric acid is ingested as the sodium salt; however, in one of the experiments here recorded, as much sodium was contained in the single dose of a mixture of sodium citrate and citric acid as was contained in the sum of the smaller doses administered by Schuck.

In other experiments in which sodium citrate was administered,

titration values of organic acid by the Van Slyke and Palmer procedure, before and after treatment to remove citrate, differed only by the amount of citrate removed. Apparently the difference is not due to removal of organic acid during the precipitation with calcium and phosphate. Until the experiment can be checked on a larger number of subjects, the differences may be attributed to individual variations in tolerance for citric acid.

#### SUMMARY

A method is described for the measurement of the buffer values of unidentified urine constituents. It may be used to detect the presence of unidentified urine constituents and will aid in their identification by indicating their dissociation constants.

From pH 5.0 to 8.0 amino acids and other unidentified urine constituents exert very little buffer action. Beyond these limits the buffer action exerted by these substances increases; amino acids are responsible for a large part of this unidentified buffer action.

Ingestion of acid or alkali causes no significant redistribution of the unidentified substances responsible for urine buffer action.

Ingestion of sodium citrate or of a mixture of sodium citrate and citric acid does not cause the excretion of significant amounts of unidentified organic acid.

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# THE UTILIZATION OF CARNOSINE BY THE DIPHTHERIA BACILLUS

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$\beta$ -Alanine has recently been shown to be essential to the growth of certain yeasts (1) and of the diphtheria bacillus (2). In the latter instance it was further shown that *l*-carnosine was also suitable, although relatively more than the calculated amount had to be supplied. Since little is known of the functions of these compounds in metabolism, it seemed worth while to examine somewhat more closely the conditions under which they exert their growth-promoting effect. In the present study, the effects on the growth of a strain of the diphtheria bacillus produced by synthetic *l*- and *d*-carnosine, and by synthetic  $\beta$ -alanine, have been compared.

The writer is indebted to Professor Vincent du Vigneaud of the School of Medicine, George Washington University for the specimens of synthetic carnosine.

## Method

To 10 cc. lots of control medium, adjusted to pH 7.4 to 7.6, one or another of the substances to be tested was added in varying amounts; the tubes were sterilized in the autoclave, inoculated with the test strain, and incubated for approximately 60 hours at 34° in a slanted position. The bacterial growth was then centrifuged out, washed with dilute acetic acid, and the relative amounts of growth estimated by means of nitrogen determinations on the washed bacterial sediment. The control medium had the composition given in Table I, the quantities noted being for 10 cc. of medium.

The hydrolyzed carnosines were prepared by autoclaving the



TABLE I  
Composition of Control Medium per 10 Cc.

	mg.		mg.
Casein-HCl hydrolysate*.....	100	MgCl <sub>2</sub> ·6H <sub>2</sub> O.....	6
<i>l</i> -Aspartic acid.....	25	CaCl <sub>2</sub> ·2H <sub>2</sub> O†.....	0.5
<i>d</i> -Glutamic acid hydrochloride.....	25	FeCl <sub>3</sub> ·6H <sub>2</sub> O†.....	0.050
<i>l</i> -Cystine.....	5	MnCl <sub>2</sub> ·4H <sub>2</sub> O†.....	0.010
Nicotinic acid.....	0.015	CuSO <sub>4</sub> ·5H <sub>2</sub> O†.....	0.002
Pimelic acid.....	0.001		cc.
Na <sub>2</sub> HPO <sub>4</sub> .....	20	Lactic acid (as Na salt)....	0.1
KCl.....	4	Ethyl alcohol.....	0.05

\* Casein was hydrolyzed by 18 hours refluxing with 6 times its weight of concentrated HCl. The excess HCl was distilled off *in vacuo*; the residue was taken up in water to 20 per cent concentration (as of the original casein) and stirred with sufficient norit charcoal to remove most of the melanin. The light yellow filtrate was made definitely alkaline before use with NaOH, and the precipitated calcium phosphate, which contains also iron and probably other metals, was filtered off.

† These salts were sterilized separately in very dilute HCl and added sterilely to the other constituents after autoclaving, thus avoiding the formation of a precipitate.

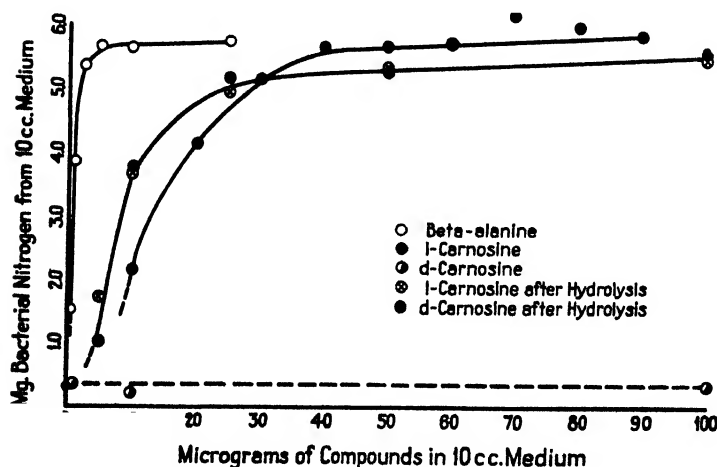


FIG. 1. Effect of  $\beta$ -alanine and carnosine on the growth of the diphtheria bacillus.

material with  $N$  HCl at a concentration of 0.1 mg. of carnosine per cc. at 15 pounds pressure for 20 minutes.

The curves of Fig. 1 show the results obtained.

#### DISCUSSION

Only the naturally occurring *l*-carnosine is utilized. It seems probable that the diphtheria bacillus is able to hydrolyze this by means of a specific enzyme, which does not act on *d*-carnosine, to *l*-histidine and  $\beta$ -alanine, and that the latter is utilized as such. This is indicated by the fact that less growth is produced by the carnosine than by the equivalent amount of  $\beta$ -alanine, which constitutes about 40 per cent of the carnosine molecule. If the organism could use the two substances directly and interchangeably, or if it had first to synthesize the peptide form, maximum growth should be produced by approximately 10 to 12 micrograms of carnosine. Actually, about 4 times that amount is required.

It is clear, also, that chemical (acid) hydrolysis of the two isomers yields equally active products. The lower maximum reached with the acid hydrolysates is probably without significance, for the experiment was not carried out on the same day as the others, and slight variations in the control solutions or in periods of incubation could well account for the difference.

These experiments offer no evidence as to the function of  $\beta$ -alanine in the metabolism of the diphtheria bacillus. The apparent synergistic effect which it exerts with nicotinic acid (2) may be fortuitous, but in view of the growing evidence that nicotinic acid is involved in the vitamin  $B_2$  complex, the observation may be not entirely without significance.

#### CONCLUSION

The diphtheria bacillus is able to obtain the  $\beta$ -alanine which it requires for growth from *l*-carnosine but not from *d*-carnosine, probably by means of enzymatic cleavage.

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# ESCHSCHOLTZXANTHIN: A NEW XANTHOPHYLL FROM THE PETALS OF THE CALIFORNIA POPPY, ESCHSCHOLTZIA CALIFORNICA

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Esters of a hitherto undescribed xanthophyll, now called *eschscholtzxanthin*, are the principal constituents of the pigment mixture found in the brilliant, golden yellow petals of the California poppy, *Eschscholtzia californica* (1-3).<sup>1</sup> Free *eschscholtzxanthin*, obtained by saponification of the esters, has the empirical formula  $C_{40}H_{54\pm 2}O_2$ . The *eschscholtzxanthin* molecule contains twelve double bonds, probably in a single conjugated system, and two hydroxyl groups.

Various synthetic esters of *eschscholtzxanthin* exhibit great variations in optical rotation; yet the wave-lengths of the absorption maxima of the esters and of the *eschscholtzxanthin* itself are identical when measured in the same solvents (Tables I and II).

<sup>1</sup> *Eschscholtzia californica* is a species subject to great variation, plants with flowers ranging from red to white having been produced (4). During the early stages of their development, the poppy petals contain considerable quantities of chlorophyll and little or no *eschscholtzxanthin*. By the time the calyx or calyptra has been burst by the expanding petals, the latter contain large quantities of *eschscholtzxanthin* and other carotenoids but no chlorophyll. Light does not seem to be essential for the formation of the pigments, because small buds placed in light-tight metal tubes develop into highly pigmented flowers. When the petals are killed with anesthetics, the pigments are not oxidized rapidly as are the carotenoids in etiolated seedlings and in young green leaves. Petals of *Eschscholtzia lemmonii*, a species closely related to *Eschscholtzia californica*, contain large quantities of *eschscholtzxanthin* as well as *zeaxanthin* and other xanthophylls. Flowers of *Papaver heterophyllum*, the wind poppy, contain only traces of carotenoid pigments. Flowers of *Dendromecon rigida* contain relatively large quantities of *neoxanthin*, a xanthophyll isolated from green leaves (see foot-note 6).

This suggests that the ester groups are located near the asymmetric carbon atoms but not in conjugation with the double bonds.

Molecular, spectral absorption coefficients of some of the esters of eschscholtzxanthin are considerably greater than those of the free eschscholtzxanthin (Table III). In this respect, eschscholtzxanthin and its esters differ from the common xanthophylls lutein, zeaxanthin, and cryptoxanthin, which exhibit molecular absorption coefficients identical with those of their esters (Table IV).

Exposure of solutions of eschscholtzxanthin to heat alters the spectral absorption properties of the pigment. This demonstrates that other colored products are formed, and it indicates that great care must be exercised during the isolation and purification of the pigment if unaltered preparations are to be obtained.

Crystals and solutions of eschscholtzxanthin and its esters absorb oxygen from the air so much more rapidly than the common carotenoids that precise determinations of the physical and chemical properties of the former compounds were made with difficulty. The rate of oxidation varies in different solvents, and, surprisingly enough, it is not increased by the presence of hemin, which has been reported by Franke to accelerate the oxidation of lycopene (5). Partially oxidized eschscholtzxanthin, which acts as an oxidizing agent, loses considerable weight when heated or illuminated, but only a trace of this is due to loss of oxygen. This observation suggests that partially oxidized carotenoids may be the source of the oxygen which has been obtained by irradiation of killed leaf material and which has been presumed to indicate the occurrence of photosynthesis.

#### EXPERIMENTAL

*Isolation of Eschscholtzxanthin. Method I*—Fresh poppy petals (8.15 kilos, or about 63,000 petals) were dried at 45–47° for 20 hours. (Drying in air at higher temperatures results in a very rapid loss of pigment.) The dried petals (1.15 kilos) were ground to pass a 20 mesh sieve and extracted with petroleum ether. This extract was concentrated and the esters present were saponified with a solution of potassium hydroxide in methanol. After the addition of water to the saponification mixture, the two layers which formed were separated, and the xanthophyll was extracted

from the petroleum ether layer with methanol (80 per cent). (Adsorption of the petroleum ether solution on a column of magnesia and siliceous earth (6) demonstrated the presence of colorless hydrocarbons, of a carotene less strongly adsorbed than  $\alpha$ -carotene, and of  $\alpha$ - and  $\beta$ -carotene (7).) The xanthophyll contained in the combined methanol solutions was extracted with ether (three 600 ml. portions) which was concentrated to about 500 ml. and cooled, whereupon a crop of purple-red crystals separated. Weight, 2.2 gm. (Crop I). By reextraction of the methanol solution of the xanthophylls and by concentration of the mother liquors of Crop I, 1.9 gm. of rather impure eschscholtz-xanthin were obtained (Crop II). Crop I was recrystallized from ethyl acetate (100 ml.) from which 0.75 gm. of large, glistening, purple-red crystals separated (Crop I-a); melting point, 183.5–184.5°; per cent of solvent lost at 84° and 1 mm., 4.9. The ethyl acetate mother liquors of Crop I-a were used for the recrystallization of Crop II, which yielded 1.25 gm. of glistening crystals (Crop II-a); melting point, 182.5–183.5°. A portion of Crop I-a (0.3 gm.) was further purified by recrystallization from acetone (40 ml.). These crystals, which were proved to be nearly homogeneous by chromatographic adsorption, were collected on a chilled filter, transferred quickly to a glass drying apparatus, and dried at 84° and 1 mm. for 1 hour. After the drying apparatus had cooled, it was filled with natural gas (methane) and small portions of the crystals were transferred to specimen tubes. These were loosely stoppered and placed in slightly larger test-tubes which were in turn quickly constricted, evacuated, and sealed. A fresh sample was used for each of the determinations of the physical and chemical properties of the pigment.

M.p., 185–186°.<sup>2</sup> Number of double bonds, 11.5, 11.7.<sup>3</sup> Logarithms of molecular, spectral absorption coefficients in ethanol, 5.073 at 445 m $\mu$ , 5.196 at 470 m $\mu$ , 5.195 at 475 m $\mu$ , 5.029 at 490 m $\mu$ , 5.085 at 500 m $\mu$ , 5.092 at

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<sup>2</sup> All melting points were determined in a Berl block of copper (8) and are corrected for the exposure of the stem of the thermometer. Open melting point tubes were used.

<sup>3</sup> The absorption of hydrogen was measured in the apparatus described by Smith (9), so modified that the material to be hydrogenated could be dropped into the solution by turning a ground glass stopper (10). The solvent was acetic acid (2 ml.) and decalin (3 ml.).

505 m $\mu$ .<sup>4</sup> Number of hydroxyl groups, 2.1, 2.2.<sup>5</sup>  $[\alpha]_{D}^{18} = +225^{\circ} \pm 12^{\circ}$  (chloroform).

C <sub>40</sub> H <sub>64</sub> O <sub>2</sub> .	Found.	C 84.11, 83.85, H 9.75, 9.60
	Calculated.	" 84.73, " 9.62
C <sub>40</sub> H <sub>66</sub> O <sub>2</sub> .	"	" 84.43, " 9.94
C <sub>40</sub> H <sub>68</sub> O <sub>3</sub> .	"	" 82.12, " 9.67

*Method II*—Fresh poppy petals (3.0 kilos) were placed in 6 times their weight of boiling water for 10 minutes. The petals were then cooled with water, pressed in a hand press, and dehydrated with two 3 liter portions of methanol (99 per cent). (Recovery of the methanol from the extracts by distillation left a residue from which about 5 gm. of crude rutin were obtained (3).) The carotenoid pigments were extracted directly from the dehydrated petals with petroleum ether, and the extracts were concentrated under reduced pressure. The oily, highly colored residue, which contained the eschscholtzxanthin esters, was treated with methanol (500 ml., 99 per cent) and cooled to 0° with an ice bath. (Repeated attempts to crystallize the esters from a variety of organic solvents were unsuccessful.) The methanol was separated from the insoluble xanthophyll esters by decantation, and the esters were saponified with potassium hydroxide dissolved in methanol. The free xanthophylls were then transferred to ether which was dehydrated with sodium sulfate in the dark at 5°. This ether solution of the xanthophylls was concentrated to about 35 ml., transferred to a 250 ml. Erlenmeyer flask with a little dioxane (20 to 25 ml.), and diluted to about 200 ml. with petroleum ether. Crystals of eschscholtzxanthin which separated were collected on a filter, washed with methanol (80 per cent), and dried in a vacuum over calcium chloride and potassium hydroxide. Yield, 2 to 3 gm. Spectral absorption coefficients, Curve III in Fig. 1, indicated that the crystalline xanthophyll was contaminated with other substances. This was confirmed by chromatographic adsorption of the pigment preparation. A num-

<sup>4</sup> All the spectral absorption coefficients were determined with the photoelectric spectrophotometer described by Smith (11).

<sup>5</sup> Active hydrogen atoms were determined with a modification of the Zerewitinoff apparatus described by Flaschentrager (12). Anisole was used as solvent.

ber of xanthophylls including flavoxanthins,<sup>6</sup> zeaxanthin, and lutein were separated from the eschscholtzxanthin in this way.

The crude eschscholtzxanthin was purified by crystallization from a variety of solvents such as acetone, ethyl acetate, and 1,2-dichloroethane. It was also purified by crystallization from pyridine and from dioxane by the addition of petroleum ether or of methanol and ethanol. These xanthophyll preparations retained from 1 to 6.9 per cent solvent, which was removed by

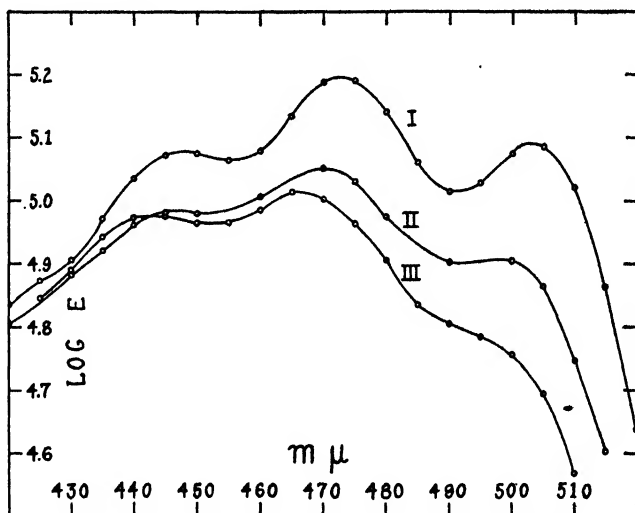


FIG. 1. Curve I, absorption spectrum of purified eschscholtzxanthin. Curve II, absorption spectrum of eschscholtzxanthin after the solution had been heated at 70° for 3 hours. Curve III, absorption spectrum of crude eschscholtzxanthin isolated by crystallization from petroleum ether (Method II). Solvent, 95 per cent ethanol.

drying the crystals at 84° and 1 mm. The eschscholtzxanthin was also purified by chromatographic adsorption upon columns composed of magnesium oxide (Micron Brand No. 2641) (6) and siliceous earth. 1,2-Dichloroethane was used as solvent. Within the limits of experimental error, the properties of each of these

<sup>6</sup> Methods for the isolation and separation of the leaf xanthophylls as well as descriptions of the properties of these pigments are to be described in a forthcoming publication of the Carnegie Institution of Washington.



various preparations of eschscholtzxanthin were identical with those of the pigment prepared as described under Method I. The molecular-spectral absorption curve of a purified preparation of eschscholtzxanthin is shown as Curve I, Fig. 1.

*Wave-Length of Absorption Maxima of Eschscholtzxanthin*—The wave-lengths of the absorption maxima of eschscholtzxanthin ( $\pm 1 m\mu$ ) in solution in various organic solvents are recorded in Table I.

*Color Reactions of Eschscholtzxanthin*—Addition of a chloroform solution of eschscholtzxanthin to concentrated sulfuric acid

TABLE I  
*Wave-Lengths of Absorption Maxima of Eschscholtzxanthin in Solution in Various Organic Solvents*

Solvent	Wave-lengths of absorption maxima		
	<i>mμ</i>	<i>mμ</i>	<i>mμ</i>
Ethanol.....	446	472	503
Isopropanol.....	446	473	503
Ethyl ether.....	446	473	503
Isopropyl ether.....	446	472	501
<i>n</i> -Heptane.....	446	472	502
Acetone.....	445	473	504
Cyclohexane.....	449	476	505
Benzene.....	458	485	516
Dioxane.....	453	480	510
Tetrahydronaphthalene.....	461	489	520
1,2-Dichloroethylene.....	455	482	512
Carbon tetrachloride.....	456	484	514
Chloroform.....	456	484	513
Pyridine.....	463	489	521
Carbon disulfide.....	475	502	536

yields a relatively stable blue color. With nitric acid, a deep blue-green color which fades rapidly is formed. A concentrated solution of eschscholtzxanthin in ether and formic acid yields a comparatively stable green color. Solutions of eschscholtzxanthin in ether do not react with concentrated hydrochloric acid to form colored products.

Solutions of eschscholtzxanthin in 1,2-dichloroethane and in chloroform react with trichloroacetic acid and with antimony trichloride, forming purple-green solutions which do not exhibit well defined absorption maxima. A solution of eschscholtz-

xanthin in dichloroethane reacts with concentrated sulfuric acid, forming a deep blue color, most of which remains in the dichloroethane.

Zinc and acetic acid in pyridine and aluminum amalgam in ether reduce eschscholtzxanthin to colorless products. Attempts to isolate known pigments from partially hydrogenated eschscholtzxanthin by means of chromatographic adsorption methods were not successful.

*Chromatographic Adsorption of Eschscholtzxanthin*—Eschscholtzxanthin, which resembles the common dihydroxyxanthophylls with respect to partition between immiscible solvents, is more strongly adsorbed than zeaxanthin and less strongly adsorbed than chlorophyll A or capsanthin on columns composed of magnesium carbonate, calcium carbonate, or sucrose when either benzene or carbon disulfide is used as solvent. The color of the adsorbed pigment varies from light pink or salmon to red-purple, depending upon the concentration of the adsorbed pigment and upon the solvents and adsorbents.

*Action of Heat on Solutions of Eschscholtzxanthin in Ethanol*—Eschscholtzxanthin (1.353 mg.) was dissolved in ethanol (100 ml.). A 3 ml. portion of this solution was diluted to 50 ml. with ethanol, and the spectral absorption coefficients were determined (Curve I, Fig. 1). Another 3 ml. portion of the original solution was placed in a 50 ml. flask and heated at 70° for 2 hours. After the solution had been cooled and diluted to 50 ml., its absorption coefficients were determined (Curve II, Fig. 1). The relatively smaller absorption at longer wave-lengths by the eschscholtzxanthin, which had been heated, demonstrates that a large proportion of the pigment had been altered. A similar alteration has been observed when solutions of the carotenes and common xanthophylls are heated.<sup>6</sup>

*Absorption Spectrum of Eschscholtzxanthin in Carbon Disulfide*—The molecular, spectral absorption coefficients of eschscholtzxanthin in solution in carbon disulfide (containing 0.5 per cent ethanol in order to prevent adsorption of the pigment on the walls of the glass vessels) are recorded in Table III. These coefficients, which are considerably different from the spectral absorption coefficients of lycopene (Table III) indicate that eschscholtzxanthin is not a dihydroxylycopene (13).

*Absorption of Oxygen by Eschscholtzxanthin*—Crystals of esch-

scholtzxanthin exposed to air absorb oxygen at a nearly constant rate until their weight has increased almost 20 per cent in 20 hours. Absorption of oxygen then decreases until, after 160 hours, the crystals have gained about 28 per cent in weight. When crystals which had been exposed to air for 44 hours and which had gained 26.5 per cent in weight were heated at  $84^{\circ}$  and 1 mm., the weight of the crystals decreased about 5 per cent. Upon reexposure to air the heated crystals did not continued to

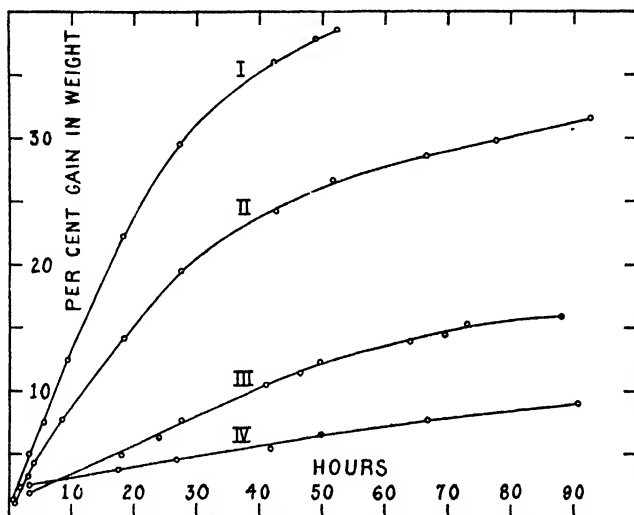


FIG. 2. Increase in weight of eschscholtzxanthin, in solution in various solvents, exposed to oxygen. Solvents, Curve I 1,4-dioxane, Curve II diacetone alcohol, Curve III diacetone alcohol containing 1 per cent dimethylaniline, Curve IV diacetone alcohol containing 10 per cent dimethylaniline.

increase in weight as rapidly as before. This indicates that decomposition had taken place during the heating.

For the investigation of the absorption of oxygen by solutions of eschscholtzxanthin, the hydrogenation apparatus described by Smith (9) was modified so that it could be filled with oxygen from a cylinder and so that a sample of the substance to be oxidized could be added to the solvent by turning a ground glass stopper (10). Absorption of oxygen was followed volumetrically, and the

apparent increase in weight of the material undergoing oxidation was calculated.

Absorption of oxygen by eschscholtzxanthin (9.08 mg.) in solution in dioxane (5 ml.) is represented by Curve I in Fig. 2. The absorption of oxygen by  $6.47 \pm 0.05$  mg. portions of eschscholtzxanthin in solution in 10 ml. portions of mixtures of solvents is represented by Curves II, III, and IV in Fig. 2.

TABLE II  
*Properties of Esters of Eschscholtzxanthin*

Ester	M. p.	$[\alpha]_{D_{25}}^{20}$ (CHCl <sub>3</sub> )	Formula	Carbon		Hydrogen	
				Found	Calculated	Found	Calculated
	°C.	degrees		per cent	per cent	per cent	per cent
Diacetate*	Decomposition 200-240	+132	C <sub>44</sub> H <sub>48</sub> O <sub>4</sub>	80.00 79.82	80.93	8.75 8.90	9.11
Dipalmitate†	100-110		C <sub>72</sub> H <sub>114</sub> O <sub>4</sub>	81.91	82.85	11.16	11.02
Dibenzoate‡	133	-142	C <sub>64</sub> H <sub>62</sub> O <sub>4</sub>	83.23 83.19	83.67	8.26 8.14	8.07
Di- <i>p</i> -nitrobenzoate§	>260	-234	C <sub>64</sub> H <sub>60</sub> O <sub>6</sub> N <sub>2</sub>		-		
Dioleate	Non-crystalline						

\* Recrystallized from carbon disulfide.

† Recrystallized from acetone.

‡ Recrystallized from acetone and ethanol.

§ Recrystallized from chloroform and acetone.

In solution in pure dimethylaniline, eschscholtzxanthin is not oxidized at a measurable rate. If large quantities of eschscholtzxanthin are added to the dimethylaniline, the crystals which remain in suspension continue to absorb oxygen slowly.

Hemin does not increase the rate of oxidation of eschscholtzxanthin in solution in dimethylaniline. Eschscholtzxanthin (1.195 mg.) in dimethylaniline (10 ml.) containing hemin (0.14 mg.) in pyridine (0.04 ml.) absorbed only 0.02 ml. of oxygen when shaken with this gas for 80 hours. In another experiment, eschscholtz-

xanthin (8.714 mg.) in suspension in dimethylaniline (5 ml.) containing hemin (1.960 mg.) and pyridine (0.2 ml.) absorbed 1.05 ml. of oxygen during constant shaking for 40 hours. This was about the same quantity of oxygen absorbed by an equal weight of

TABLE III

*Molecular, Spectral Absorption Coefficients of Eschscholtzxanthin, of Esters of Eschscholtzxanthin, and of Lycopene; Solvent, Carbon Disulfide*

Wave-length	Eschscholtzxanthin	Eschscholtzxanthin diacetate	Eschscholtzxanthin dipalmitate	Eschscholtzxanthin dibenzoate	Eschscholtzxanthin di-p-nitrobenzoate	Lycopene (Smith (11))
$m\mu$	$\log E$	$\log E$	$\log E$	$\log E$	$\log E$	$\log E$
430	4.528	4.539	4.545	4.526	4.564	4.441
435	4.602					4.502
440	4.660	4.679	4.669	4.671	4.692	4.574
445	4.702					4.632
450	4.736	4.749	4.748	4.756	4.765	4.668
455	4.776	4.796				4.699
460	4.836	4.855	4.834	4.854	4.870	4.748
465	4.906	4.925			4.937	4.831
470	4.958	4.972	4.960	4.981	4.986	4.902
475	4.972	4.996	4.973	5.003	5.010	4.946
480	4.973		4.969		5.011	4.950
485	4.981	4.991	4.973		5.012	4.938
490	5.008		4.986			4.946
495	5.059		5.060		5.085	5.000
500	5.095					5.073
505	5.100	5.128	5.106	5.137	5.142	5.123
510	5.063					5.121
515	5.002	5.026			5.059	5.083
520	4.954	4.972	4.949	4.990	5.002	5.020
525	4.936	4.954	4.942	4.964		4.961
530	4.958			4.985		4.941
535	4.992	5.023	5.001	5.022	5.022	4.987
540	4.989	5.030	5.000	5.035	5.034	5.058
545	4.929	4.957	4.935		4.988	5.091
550	4.799	4.851	4.810	4.883		5.044
555	4.609			4.702	4.681	

eschscholtzxanthin in suspension in pure dimethylaniline. Hemin and hemin with pyridine did not absorb oxygen when dissolved in dimethylaniline. These results differ markedly from the observation of Franke (5); namely, that the less reactive carotenoid

lycopene (5 mg.) dissolved in dimethylaniline (2 ml.) containing hemin (0.1 mg.) and pyridine (0.04 ml.) absorbed about 6 ml. of oxygen in 3 hours.

*Evolution of Oxygen by Partially Oxidized Eschscholtzxanthin*—Treatment of partially oxidized eschscholtzxanthin with a solution of leucoindigo carmine in an atmosphere of carbon dioxide free from oxygen results in a rapid oxidation of the reduced dye to the colored or oxidized form." A suspension of partially oxidized eschscholtzxanthin treated with a solution of 3-amino-phthalylhydrazide and hemin in dilute sodium carbonate solution

TABLE IV  
*Logarithms of Molecular, Spectral Absorption Coefficients of Xanthophylls and Their Esters in Solution in Carbon Disulfide*

Wave-length	Lutein	Lutein acetate	Lutein p-nitrobenzoate	Zeaxanthin	Zeaxanthin p-nitrobenzoate	Cryptoxanthin	Cryptoxanthin p-nitrobenzoate
m $\mu$	log E	log E	log E	log E	log E	log E	log E
430	4.675	4.674	4.662	4.619	4.630	4.612	
440	4.819	4.820	4.809	4.737	4.749	4.730	4.733
450	4.866	4.858	4.850	4.839	4.849	4.836	4.831
460	4.916	4.900	4.890	4.884	4.888	4.886	4.881
470	5.029	5.032	5.019	4.960	4.974	4.960	4.953
480	5.012	5.021	5.002	5.021	5.031	5.033	5.031
490	4.925	4.919	4.897	4.989	4.988	5.015	5.010
500	4.979	4.979	4.960	4.950	4.947	4.968	4.959
510	4.978	4.994	4.968	4.970	4.982	4.986	4.976
520	4.756	4.764	4.720	4.918	4.922	4.962	4.942
530	4.339			4.719	4.686	4.792	4.760
540				4.333			4.391

gives rise to a distinct luminescence. This indicates the presence of peroxides (14). When partially oxidized eschscholtzxanthin, in a modified Faraday tube containing leucoindigo carmine as a reagent for oxygen, is illuminated with a photoflood light, only traces of oxygen are evolved.

*Esters of Eschscholtzxanthin*—The esters were prepared with acid chlorides in the presence of pyridine and crystallized by the addition of methanol or of acetone and methanol to the reaction mixture. Properties of the recrystallized esters are summarized in Table II. None of the esters contained free hydroxyl groups

(Zerewitinoff). The wave-lengths of the absorption maxima of each of the esters were the same as those of free eschscholtzxanthin ( $\pm 1 m\mu$ ) when determined in the same solvents (Table I). The molecular, spectral absorption coefficients are reported in Table III.

*Molecular, Spectral Absorption Coefficients of Xanthophylls and Their Esters*—The spectral absorption coefficients of lutein, zeaxanthin, and cryptoxanthin were compared with those of their esters given in Table IV. Carbon disulfide was employed as solvent. These results demonstrate that the spectral absorption coefficients of the common xanthophylls are nearly identical with those of their esters. Conversely, these observations provide an accurate confirmation of the molecular weights of the xanthophylls. They also indicate that the formation of colored esters or derivatives, the absorption coefficients of which can be accurately determined, may be made the basis of methods for the determination of the molecular weights of acids, alcohols, amines, etc.

It is a pleasure to acknowledge the advice and helpful suggestions of Dr. H. A. Spochr and Dr. James H. C. Smith.

#### SUMMARY

Eschscholtzxanthin, a new xanthophyll having the empirical formula  $C_{40}H_{54\pm 2}O_2$ , has been isolated in relatively large quantities from the petals of the California poppy. This xanthophyll, which is oxidized extremely rapidly and which is altered by the action of heat upon its solutions, contains twelve conjugated double bonds and two hydroxyl groups. The optical properties of eschscholtzxanthin and its esters have been compared with the optical properties of the common xanthophylls and their esters.

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## SURFACE DENATURATION OF EGG ALBUMIN

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In a study on the conditions governing the coagulation of protein by shaking, Wu and Ling (1) found that the rate of coagulation of egg albumin by shaking was maximum at its isoelectric point, and that at a constant pH the rate of coagulation was proportional to the number of shakes but independent of the concentration of albumin. They found further that coagulation by shaking, unlike coagulation by heat, was not divisible into two stages, denaturation and flocculation.

In a recent paper Bull and Neurath (2), apparently not aware of the work of Wu and Ling, reported a study of surface denaturation of egg albumin. They have confirmed in general the findings of Wu and Ling, but in two points their conclusions were different.

First, Bull and Neurath found that the higher the protein concentration the lower is the rate of denaturation, if the velocity constant was calculated on the basis of monomolecular reaction. While this finding was not incorrect, they have missed the real point. In Table I of their paper it will be noted that for the first solution the amount of albumin coagulated in  $4\frac{1}{2}$  hours was 0.39 gm., and for the second solution the amount coagulated in 4 hours was 0.34 gm. or by interpolation 0.38 gm. in  $4\frac{1}{2}$  hours. The rates of coagulation were, therefore, the same for these two solutions, although the concentration of albumin in the first solution was nearly twice as high as that in the second.

We have repeated the experiments of Wu and Ling on the kinetics of coagulation by shaking, using 125 ml. and 50 ml. bottles. The albumin left in the solution after shaking was determined by a micro-Kjeldahl method and titration instead of the colorimetric method of Wu and Ling. We have confirmed their finding that the rate of coagulation is proportional to the number of

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shakes and, within wide limits, independent of the concentration of albumin. The curves shown in Fig. 1 are unquestionably linear.

Second, Bull and Neurath state that, "The process of surface coagulation seems to consist of at least two reactions," comparable with the denaturation and flocculation in heat coagulation. In other words, a protein may be denatured by shaking but

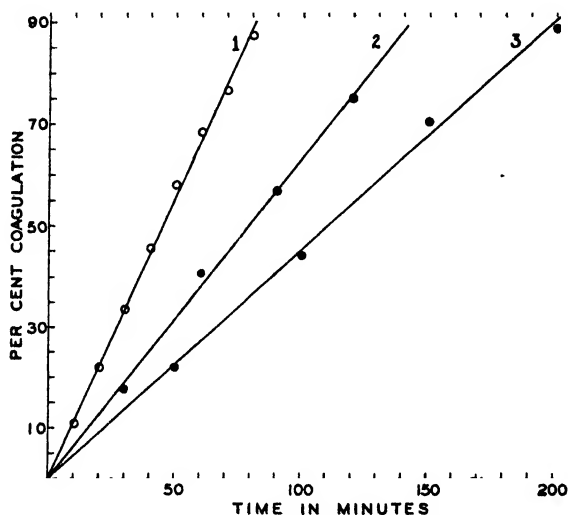


FIG. 1. Kinetics of coagulation of egg albumin by shaking. Curve 1, 10 ml. of 2.051 per cent egg albumin solution + 5 ml. of acetate buffer solution, pH 4.8, + 5 ml. of  $H_2O$ ; capacity of bottle, 125 ml.; temperature,  $28^\circ$ . Curve 2, 10 ml. of 0.959 per cent egg albumin solution + 5 ml. of acetate buffer solution, pH 4.8, + 3 ml. of  $H_2O$ ; capacity of bottle, 50 ml.; temperature,  $27^\circ$ . Curve 3, 10 ml. of 1.060 per cent egg albumin solution + 5 ml. of acetate buffer solution, pH 4.8, + 3 ml. of  $H_2O$ ; capacity of bottle, 50 ml.; temperature,  $12^\circ$ .

remains in solution, if the solution is acid or alkaline. When the solution is neutralized, the denatured protein will precipitate out. The conclusion of Bull and Neurath was based on the finding that when an albumin solution was shaken at a pH other than the isoelectric point the amount of precipitate obtained was greater if the solution was brought to pH 4.8 before filtration than when it was not so adjusted.

Wu and Ling showed that albumin solutions which were sufficiently acid or alkaline to remain clear after shaking gave no precipitate or turbidity when the pH was brought to 4.8. In fact, when a solution was shaken at a pH not sufficiently acid or alkaline to cause denaturation on standing, the coagulated protein filtered off, and the filtrate brought to pH 4.8, not a trace of turbidity was obtained. This finding is a conclusive proof that coagulation by shaking is not separable into two stages.

It occurred to us that the results of Bull and Neurath may be explained by adsorption. Proteins are most easily adsorbed at the isoelectric point. If some natural albumin is carried down by the coagulated albumin, the amount carried down must be greater if the solution is brought to pH 4.8 before filtration. An experiment designed to test this point showed that such an adsorption did occur, but the amount of adsorption was not enough to account for such large differences between the neutralized and the unneutralized solutions as Fig. 2 in Bull and Neurath's paper indicates.

Wu and Ling conducted their experiment at room temperature (25°), while Bull and Neurath carried out their experiments at 2°. Wu and Ling have shown that the temperature coefficient of surface coagulation for 10° is 1.09. The rate of coagulation should, therefore, be somewhat slower at 2° than at 25°, but the difference in temperature should not alter the nature of the coagulation process.

The technique of shaking which Bull and Neurath used was the same as that of Wu and Ling. The form of shaking apparatus, the number of oscillations per minute, and the length of the stroke were all about the same. But there was one important difference. Wu and Ling used 125 ml. narrow mouth bottles, while Bull and Neurath used 70 ml. wide mouth bottles. Wu and Ling have shown that for a given volume of solution the rate of coagulation depends to a large extent on the size of the bottle. Below 250 ml., the smaller the bottle the slower the rate of coagulation. In the experiment of Wu and Ling all the egg albumin in 10 ml. of a 1 per cent solution was completely coagulated in about 30 minutes. In the experiments of Bull and Neurath the time required to remove all the coagulable albumin from 25 ml. of a 1.10 per cent solution was about 12 hours.

Wu and Ling have shown that crystalline egg albumin is completely coagulable by shaking, while conalbumin is not coagulable. For this reason they used highly purified egg albumin which was practically all coagulable by shaking. Bull and Neurath used two kinds of albumin preparations, one of which was only 72.6 to 78.2 per cent, while the other was only 89.3 to 91.8 per cent surface-coagulable. The first preparation contained 14.2 to 20.0 per cent non-heat-coagulable protein; the second contained 4.3 per cent non-heat-coagulable protein. The albumin preparations which Bull and Neurath used were, therefore, quite impure.

The long period of shaking in Bull and Neurath's experiment exposed the albumin to denaturation by acid and alkali and even to bacterial action. This, together with the fact that their albumin preparations were impure, probably explains the difference between their findings and those of Wu and Ling.

#### SUMMARY

The finding of Bull and Neurath that surface coagulation of egg albumin consists of two stages is incorrect. Within the limits studied, the rate of coagulation is independent of the concentration of albumin and does not decrease with concentration as Bull and Neurath have stated. With these two exceptions, the work of Bull and Neurath confirmed the findings of Wu and Ling.

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# THE CARBOHYDRATE METABOLISM OF BRAIN

## VI. ISOLATION OF GLYCOGEN\*

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Although the presence of glycogen in brain was accepted by Pflüger (1905), more recent work has done little to strengthen the evidence submitted as early as 1887 by Cramer, and a number of publications even raise doubt concerning the existence of glycogen in brain or its identity with liver glycogen.

Pavy (1881), credited by Pflüger as the first to find glycogen in brain, applied to brain a procedure used for isolating liver glycogen, and obtained an alcohol-insoluble material which on acid hydrolysis yielded a reducing substance. Since a number of compounds other than glycogen (*e.g.*, cerebrosides) behave similarly, this evidence is unsatisfactory. Cramer (1887), however, obtained a substance which undoubtedly contained glycogen. His product prepared according to the Brücke-Külz quantitative procedure (see Pflüger) was opalescent in aqueous solution, reacted like liver glycogen with iodine solution, and yielded a reducing substance after treatment with saliva.<sup>1</sup> Takahashi (1925) attempted to isolate glycogen from brain, but the yield of sugar after hydrolysis indicated that less than a fourth of his material could have been glycogen, and the color which developed on addition of iodine was not identical with that obtained with liver glycogen. Holmes and Holmes (1926) applied the Pflüger procedure to brain after extracting cerebrosides, and obtained in the hydrolysate of the alcohol-insoluble material an osazone iden-

\* Aided by a grant from the Rockefeller Foundation.

<sup>1</sup>Quoted from Pflüger (1905). Since the original article is not available here, we are unable to state what evidence Cramer submitted as to the purity of his compound.

tical in appearance with that of glucosazone.<sup>2</sup> Tanaka (1929) concluded, on the basis of histological studies, that glycogen was present in brain, judging both from its microchemical and biological properties. Sato (1930,b) distinguished two types of glycogen by staining methods, and claimed that besides the true glycogen there is also present in the central nervous system a modification which gives no iodine reaction, but otherwise behaves like glycogen.

The validity of evidence based on histological studies must, however, be questioned. Naka (1930) attempted to standardize his histological method for quantitative purposes by comparison with a chemical procedure. On examining the brains of 72 human cadavers by this method he found the largest amounts of glycogen in two brains removed 26 hours after death (Naka, 1930), whereas we have shown that glycogen disappears almost completely from brain within 2 hours after death (Kerr and Ghantus, 1937). We must therefore conclude that by his method Naka actually determined something other than glycogen. Consequently Best's carmine method (which Naka used) appears to stain material other than glycogen, and is unreliable as a means of distinguishing between traces and the normal content of glycogen in nerve tissue. Sato (1930,a) states that the color reagent of Best and the iodine reaction of Langhans are not specific for glycogen. Nielsen, Okkels, and Stockholm-Borresen (1932) state that these staining procedures cannot be relied upon when the concentration of glycogen in the tissue is less than 0.3 per cent. In mammalian brain the concentration is less than 0.15 per cent (Kerr, 1936). De Haan (1922) earlier reviewed the uncertainties connected with the staining reactions for glycogen.

Gerard (1932) states: "Brain glycogen however appears to have been thoroughly identified with the liver substance, yet it is also inactive." Of the nine papers referred to in support of this conclusion, in five (those of the Japanese workers) conclusions were based on histological evidence (Naka, 1930; Sato, 1930,a,b; Tanaka, 1929). In two of the other investigations (Holmes, 1929; Jungmann and Kimmelstiel, 1929), it was concluded that

<sup>2</sup> Holmes (1929) also prepared an osazone in the same way from material secured from the ganglia of crab nerve and found its crystalline form and melting point identical with that of glucosazone.

the material measured by the Pflüger procedure was glycogen because autolysis resulted in a decrease of this substance and a corresponding increase of lactic acid. Reference has already been made to the studies in which direct attempts were made to isolate either the glycogen or the osazone of the hydrolysate (Holmes and Holmes, 1926; Holmes, 1929; Takahashi, 1925).

The apparent inactivity of the so called glycogen of brain, referred to by Gerard, under conditions which cause a marked decrease of glycogen in liver, leads one to question the identity of the material from the two sources. There appeared to be sufficient uncertainty, therefore, concerning the existence of glycogen in brain to make desirable an attempt to isolate the substance.<sup>3</sup>

#### EXPERIMENTAL

In our first attempts to prepare glycogen from brain the products obtained were grossly contaminated with lipoidal material. Efforts to remove this completely by means of boiling alcohol, chloroform, and ether were unsuccessful, but pure products were finally obtained by combining the use of these solvents with the principles used by Somogyi (1934) for the purification of glycogen. Five preparations have been made, each of them free from nitrogen and phosphorus. Various procedures were followed, but the essential steps are included in the description which follows, of one of the experiments.

#### *Method*

About 500 gm. of brain are required for the preparation of 150 mg. of pure glycogen. The brains of twelve dogs are excised under amytal anesthesia and rapidly crushed<sup>4</sup> with a potato masher in a nickel-plated vessel containing 60 per cent KOH solution (1 cc. of KOH per gm. of brain). The mash is transferred to a large flask, 2 volumes of ethyl alcohol are added, and the tissue brought

<sup>3</sup> The studies on brain glycogen recently published from this laboratory (Kerr, 1936; Kerr and Ghantus, 1936, 1937; Kerr, Hampel, and Ghantus, 1937; Kerr and Antaki, 1937) were undertaken only after we had isolated the substance. This publication was delayed until further purification of the product had been accomplished.

<sup>4</sup> The interval between excision from the living animal and fixation in the alkali should not exceed 10 seconds, since glycogenolysis proceeds rapidly (Kerr and Ghantus, 1937).



into solution by heating on a water bath for half an hour. After standing overnight, the supernatant fluid is removed by siphoning and centrifuging. The residue is next extracted four times<sup>5</sup> with a boiling mixture of methyl alcohol (80 parts) and chloroform (20 parts) to remove the major portion of cerebrosides. The dried residue is heated in the water bath for 3 hours in 30 per cent KOH to decompose certain nitrogenous material which otherwise clings to the glycogen.<sup>6</sup> After the insoluble material is centrifuged off, the glycogen is precipitated by 0.5 volume of alcohol, centrifuged, and washed with 95 per cent alcohol. The crude glycogen is dissolved in 10 cc. of water and most of the remaining impurities are separated by acidifying with HCl to the turning point of Congo red and then adding slowly 0.5 volume of alcohol and centrifuging. Glycogen is precipitated from the supernatant liquid by increasing the alcohol concentration to 45 per cent.

If at this point the final steps of purification are carried out as recommended by Somogyi (1934) (*i.e.*, two more precipitations in acid alcohol, followed by washing),<sup>7</sup> the product after desiccation may be partly insoluble, and will contain as much as 0.1 per cent of nitrogen. Hence the glycogen should again be precipitated by 0.5 volume of alcohol from 4 N alkaline solution, washed with alcohol, and reprecipitated in acid solution with 0.8 volume of alcohol. After two more reprecipitations in acid alcohol as described by Somogyi (1934), the glycogen is washed with alcohol and ether and dried *in vacuo* over  $P_2O_5$  at 70°.<sup>8</sup>

<sup>5</sup> In other experiments the separation of lipids was made more complete by as many as ten extractions with the methyl alcohol-chloroform mixture, followed by chloroform alone, and ether, but the yield of glycogen was low. The final traces of lipids are more easily removed in the subsequent precipitations by acid alcohol.

<sup>6</sup> The digestion with KOH may be introduced at the very beginning, after crushing the brain in alkali, but more silica is introduced owing to the necessity of using a larger flask.

<sup>7</sup> The alcohol and ether from this point on should be redistilled, as recommended by Somogyi (1934).

<sup>8</sup> The water content of the air-dried preparation is greater than the 10 per cent required for the monohydrate. Values from 11.1 to 14.3 per cent were obtained on drying to constant weight over  $P_2O_5$  *in vacuo* at 70°. Drying over  $CaCl_2$  at atmospheric pressure resulted in preparations containing 8.1 to 8.7 per cent of water. On evacuation the water content fell to 6.9 per cent, but the half-hydrate which Slater (1924) states is formed

The best yield obtained when the brains were excised and crushed as described above was 36 mg. of anhydrous glycogen per 100 gm. of brain. In another experiment the brains were frozen *in situ* with liquid air (Kerr, 1935) and the powdered tissue thrown into KOH solution. In this case the yield was 43 mg. of glycogen per 100 gm. of brain. Quantitative studies show that dog brain frozen *in situ* contains from 77 to 130 mg. of glycogen per 100 gm. (Kerr, 1936). The losses in the isolation procedure are due chiefly to the low concentration of alcohol used in the various precipitations for the sake of separating other contaminating substances, and partly to adsorption during the first precipitation of impurities by 33 per cent alcohol in acid solution.

*Analysis*<sup>9</sup>—The material was dried in a high vacuum at 100°.

5.622 mg. gave 9.075 mg. CO<sub>2</sub>, 3.000 mg. H<sub>2</sub>O, 0.010 mg. ash

(C<sub>12</sub>H<sub>30</sub>O<sub>10</sub>)<sub>n</sub>. Calculated. C 44.42, H 6.22

Found.<sup>10</sup> " 44.10, " 5.98

Phosphorus and nitrogen were not detected. Other preparations have been made free from ash.

The specific rotation of a solution 1 per cent in concentration was  $[\alpha]_D^{20} = +195.6^\circ$ , with a probable error of 0.7°.

For the sake of comparison, three specimens of glycogen prepared from dog liver by Somogyi's method (1934), each in 1 per cent solution, were studied under the same conditions. The results were +196.1°, +194.3°, and +195.2°. Gatin-Gruzewska (1904) observed a specific rotation of +196.57° as the average for four specimens of carefully purified glycogen prepared from dog liver and horse muscle, and free from nitrogen and ash.

*Properties*—The product is indistinguishable from liver glyco-

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under these conditions was not obtained, even with liver glycogen. A possible explanation for the high water content may be found in the observation of Sahyun and Alsberg (1930) that ether tends to dehydrate the surface of the glycogen particles but leaves the interior wet, thus making subsequent drying incomplete. We were unable to confirm the statement of Barbour (1929-30) that the monohydrate may be obtained by drying glycogen in an open dish over boiling water. Under these conditions loss of water occurs within a few minutes and continues for hours. After 9 hours the product contained 3.6 per cent of water.

<sup>9</sup> Performed by Dr. I. A. Schoeller, Berlin.

<sup>10</sup> Corrected for ash content by assuming the sample to be 5.612 mg.

gen prepared by Somogyi's method (1934). It dissolves readily in water to form a transparent solution, which is opalescent in reflected light. This when treated with iodine solution gives a Burgundy red color identical with that obtained with a solution of liver glycogen. The red color disappears if the solution is heated, and returns when cooled.

The identity of the compound with liver glycogen is partially shown by the method of preparation and purification. The compound is not destroyed by heating with strong alkali. It is precipitated by 0.5 volume of alcohol from alkaline but not from acid solutions. Alcohol does not precipitate the compound from pure aqueous solutions unless electrolytes are present.

Hydrolysis of the compound in solution by means of  $N$  HCl in a boiling water bath for 2 hours yielded a completely fermentable reducing sugar equivalent to 107.2 mg. of glucose per 100 mg. of the original anhydrous material, as measured by the Shaffer-Somogyi procedure (1933). Under the same conditions liver glycogen gave 106.6 mg. per cent. Kerly (1930) found that 94 per cent of the theoretical yield (111.1 mg.) can be obtained on acid hydrolysis, and therefore recommended the use of the factor 0.957 in place of the customary 0.927 (Nerking, 1901) to convert sugar found to anhydrous glycogen. According to our results with liver glycogen the factor would be 0.938. The specific rotation of the sugar in the hydrolysate was that of glucose within the limits of error of the determination.<sup>11</sup>

From the neutralized hydrolysate an osazone was obtained identical in crystalline form with phenylglucosazone. The osazone (once recrystallized) melted simultaneously with glucosazone between 204.5–206.5° (uncorrected). When mixed with an equal part of glucosazone, the melting point was 201–203°.

#### SUMMARY

A polysaccharide free from nitrogen, phosphorus, and ash, prepared from dog brain, was found to be indistinguishable from liver glycogen in respect to elementary composition, specific

<sup>11</sup> For this determination a solution of only 0.5 per cent concentration was available. The value obtained was  $[\alpha]_D^{20} = +51.3^\circ$ , with a probable error of 1.1°.

rotation, quantity of glucose produced on acid hydrolysis, precipitation from solution by alcohol, reaction with iodine, etc.

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## THE MECHANISM OF CYTOCHROME ACTION\*

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The experiments described here are a continuation of work described in an earlier paper (1) in which the reduction potentials at which cytochrome C preparations changed from the oxidized to the reduced form were studied and an estimate of the  $E'_0$  values at several pH values were made.<sup>1</sup> The cytochrome preparations there described were found to be associated with a colorless phosphorus-containing component, the relation of which to the biological activity of the pigment is discussed in the present paper.

A colorless solution was prepared by treating with 8 per cent trichloroacetic acid the solution of cytochrome C prepared from Fleischmann's yeast by the second method of Keilin (3). On removal of the acid with ether, neutralization of the remaining solution, precipitation with barium,<sup>2</sup> lead, or copper salts, and regeneration, there is obtained a white water-soluble solid, giving no Molisch reaction. The yield is 20 mg. per kilo of pressed

\* The data in this paper include material from a thesis submitted by the author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University.

<sup>1</sup> The disagreement of these findings with those published by Green (2) is probably largely due to the effect of associated substances, not including iron, on the  $E'_0$  potentials of the basic hemin compound. The final determination of these values must await the attainment of cytochrome C preparations demonstrably free of associated substances. Physiologically, the important  $E'_0$  values are those of the compound (probably in complex combination, as shown here) as it exists in the cell. Some evidence was presented in the previous paper that the  $E'_0$  values determined there were those of the pigment as it exists in the cell.

<sup>2</sup> The statement in the previous paper (1) that the compound is not precipitated by barium salts is an error; in the experiments there reported precipitation was attempted at too low a level of pH.

yeast. Analysis of one such preparation after precipitation with barium chloride, regeneration, and two subsequent precipitations with lead acetate showed C 12.6, H 1.8, N 4.18, P 6.54, Pb 50.6. As, however, no consistent trends in the composition after repeated precipitations were observed, investigation of this product was abandoned.

Cytochrome C preparations prepared in the course of the above experiments were tested by addition, to the system used by Harrison (4), of the following: indophenol oxidase (washed minced sheep heart), glucose dehydrogenase (from liver by the second method of Harrison (5)), cozymase (from yeast, always accompanied by large amounts of flavin), glucose.

The oxygen uptake was measured in the Warburg apparatus at 37°. Catalytic activity was considered present if the oxygen uptake of the system indophenol oxidase-cytochrome C-glucose dehydrogenase-coenzyme-glucose exceeded that of the same system without cytochrome by 50 per cent.

It was found that cytochrome C preparations, including several from American (Fleischmann's) and two from Delft bakers' yeast, prepared according to Keilin's second method were inactive. These findings, which are contrary to those of Harrison (4), are thought to be due to differences in the amount and kind of kieselguhr used and their effect on the removal of an inhibiting factor. In one experiment 70 per cent of the phosphorus present was separated from the pigment by adsorption of the latter on kieselguhr, washing with water, and elution with dilute alkali and sodium hydrosulfite.

It was found that by dissolving such inactive cytochrome preparations in the minimum amount of sodium hydroxide, adsorbing on permutit, washing with water, eluting with 5 per cent ammonia, and removing the latter by evaporation *in vacuo* they became catalytically active.<sup>3</sup> At the same time about half of the

<sup>3</sup> On the pigment solution eluted from permutit and evaporated free of ammonia were determined the nitrogen, phosphorus, iron (by photometric estimation of the  $\alpha, \alpha'$ -dipyridyl complex), and absorption spectrum in the visible region. Dry weights were not determined, as silica was present. Analyses of two such samples are given, the figures representing atomic ratios of nitrogen and phosphorus to iron. The  $k$  values (maximum peak values at the wave-lengths given) were calculated by the equation  $k =$

phosphorus originally present was separated from the pigment and appeared in the water used to wash the permutit prior to elution.

That the fraction removed by the permutit treatment exerts an inhibitory action<sup>4</sup> on the dehydrogenase-coenzyme substrate reaction in the presence of methylene blue is shown in Table I.

The separation of cytochrome C by Keilin's method from American (Fleischmann's) yeast is made difficult by the fact that, after plasmolysis, boiling in water, and cooling, it settles and filters much more slowly than Delft yeast, and must be kept cold during filtration or pigment is lost. Active preparations can conveniently be prepared from Fleischmann's bakers' yeast by liquefying it with glucose, passing in steam until the mixture foams, and filtering as it cools to room temperature on Buchner funnels. The resulting solution is passed through a column of permutit which is washed successively with water, 2 per cent acetic acid, and again with water. Elution with 5 per cent ammonia and removal of the latter by evaporation *in vacuo* gives a clear solution of cytochrome C which is active. Dialysis in viscose tubes may be used for further purification.

Cytochrome prepared by both methods gave identical results. The oxygen uptakes of the complete system were much lower than in those described by Harrison (4), though the cytochrome used

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$(1/cd) \ln (I_0/I)$ , where  $c$  = gm. atoms of Fe,  $d$  = tube length in cm.,  $I_0$  and  $I$  = intensities of incident and transmitted light respectively.

N	P	$k$	
174	28.2	0.405	at 5373 Å.
202	16.2	0.317	" 5467 "

The absorption spectrum of the reduced pigment from 5000 to 5700 Å., both before and after the permutit treatment, parallels exactly that obtained by Dixon, Hill, and Keilin (6), except that the spectrum is shifted 50 to 150 Å. toward the violet. The absorption coefficient,  $k$ , referred to iron concentration, varied from one-half to two-thirds of the values obtained by Dixon, Hill, and Keilin (6). This fact probably denotes contamination of the preparations with iron, but this is not believed to affect the catalytic properties of the preparations because of the well known ability of cell components to bind inorganic iron and render it catalytically inactive.

<sup>4</sup> It is conceivable that the failure of Ogston and Green (7) to confirm the findings of Harrison was due to the presence of such an inhibitory substance.



in each experiment usually represented more yeast. Controls showed that glucose was the chief substance being oxidized. It was found that cytochrome C in the absence of indophenol oxidase caused no appreciable oxygen uptake (Table II). It was, however, observed that two preparations of cytochrome which became precipitated in a finely divided state during the course of the experiment, apparently being adsorbed on unremoved silica, displayed catalytic activity in the absence of indophenol oxidase and not in its presence.

In considering the mechanism of cytochrome C action in living cells it is to be remembered that in the yeast cell the cytochrome appears to be attached to the solid substance of the cell, being removed only by fairly strong alkalinity or by reducing agents. Also, whenever in artificial systems neutral cytochrome C solutions are shaken with heart muscle suspension, the cytochrome is always rapidly adsorbed on the solid muscle substance.

The possible existence of a labile complex between cytochrome C and one or more phosphorus compounds indicated above suggested that cozymase might be bound in a similar fashion. This was now shown to be the case.

The neutral cytochrome solutions were shaken with the minced sheep heart (indophenol oxidase) and then centrifuged, when the pigment was thrown down, adsorbed on the muscle particles. Suspensions of these proved to be active without addition of further indophenol oxidase or cytochrome.<sup>5</sup> When such a suspension was stirred with a neutral solution of cozymase (from yeast) and centrifuged, the cozymase remained adsorbed and active, as shown by the activity of the suspension in the presence of glucose dehydrogenase and glucose without further addition of cozymase. When, however, the minced muscle was stirred and centrifuged, first with cozymase solution and then with the cytochrome C solution, the resulting particles were not catalytically active without further addition of cozymase.

The activity obtained in these experiments was not due to coenzyme contained in the fluid occluded by the precipitate after

<sup>5</sup> This procedure sometimes caused cytochrome solutions prepared by Keilin's method to show activity, although they were inactive when tested in the ordinary way. This is undoubtedly due to preferential adsorption of the cytochrome, the inhibitory material remaining in the supernatant fluid.

centrifugation. A volume of the supernatant fluid equal to the volume of the precipitate (and hence greater than that of the occluded fluid) was added to an indophenol-oxidase-cytochrome precipitate centrifuged from distilled water, and the activity compared with that of the parallel precipitate from a coenzyme solution but without the added supernatant fluid. The results are recorded in Table III.

The results described above, demonstrating that insoluble particles of washed heart muscle (the indophenol oxidase preparation) adsorb cytochrome and cozymase, forming a catalytically active product, seem to throw light on the relationships between the respiratory ferment and cytochrome C and the relation between the water-soluble enzymes and those which are part of the insoluble cell substance or are bound to it. They suggest the probability of the formation of an indophenol oxidase-cytochrome C-cozymase "complex," or an adsorption of cytochrome and cozymase on the surface of the solid particles (of indophenol oxidase) as part of the mechanism of activation. The fact that the indophenol oxidase preparation must be treated with the cytochrome solution before, rather than after, treatment with cozymase, suggests (but does not prove) that the cozymase may be bound to the adsorbed cytochrome rather than to the oxidase.

The oxygen uptakes in these experiments are small compared with those found in other comparable systems such as equally concentrated mixtures of succinic dehydrogenase and its substrate, and it is not believed that glucose dehydrogenase is normally responsible for a large part of the oxygen uptake of either yeast or muscle. What is believed may be important is the mechanism demonstrated here. Attempts are now being made to show its action in other systems.

#### EXPERIMENTAL

Indophenol oxidase was prepared from sheep heart by the method of Keilin (3) and suspended in 0.25 M phosphate buffer, pH 7.4. All preparations used brought about a marked darkening of *p*-phenylenediamine in neutral solution on shaking a minute or two with air.

The glucose dehydrogenase was prepared as a dry powder, free of coenzyme, from acetone liver by the method of Harrison (5).

The strength of the freshly dissolved, neutralized, and filtered or centrifuged preparations was such that 1.00 cc. decolorized 1.00 cc. of 1:5000 methylene blue solution within 3 to 10 minutes in the presence of glucose and cozymase (from yeast) and required more than 20 minutes in the absence of either.

The cozymase was prepared from Fleischmann's bakers' yeast<sup>6</sup> by one of several methods usually involving precipitation with phosphotungstic acid after preliminary precipitation with lead or barium. The cozymase used in each experiment was at least enough to give a maximal rate of decolorization of methylene blue in the presence of glucose by 1.00 cc. of glucose dehydrogenase solution.

TABLE I

*Inhibitory Effect of Fluid Remaining after Adsorption of Cytochrome on Permutit*

Tube No.....	1	2	3	4
Glucose dehydrogenase, cc.....	0.5	0.5	0.5	0.5
Methylene blue, cc.....	0.5	0.5	0.5	0.5
2 M glucose, cc.....	0.15	0.15	0.15	0.15
Cozymase solution, cc.....	0	0.15	0	0.15
"Washing," cc.....	0	0	0.5	0.5
Buffer, cc.....	1.0	1.0	1.0	1.0
Time of decolorization, min.....	17	3.5	17	7

Indophenol oxidase preparations with cytochrome adsorbed on the particles were prepared by mixing indophenol oxidase suspensions with cytochrome solutions, neutralizing to litmus, allowing to stand 15 to 60 minutes, centrifuging, discarding the supernatant fluid, and shaking the suspension with enough phosphate buffer to give the original volume. Such preparations are denoted hereafter indo-cyto.

Methylene blue, 1:5000 in water, was used as a control of the glucose dehydrogenase activity.

The glucose solutions used were boiled before use.

The buffer solution is 0.25 M phosphate, pH 7.4, in all experiments.

<sup>6</sup> The author wishes to express his gratitude to Dr. C. N. Frey of The Fleischmann Laboratories for generous supplies of yeast.

TABLE II  
*Catalytic Effect of Cytochrome*

Vessel No.	Series A						Series B					
	1	2	3	4	5	6	1	2	3	4	5	6
Indo, cc	1	0	1	1	0	0	1	0	1	1	0	0
Cyto, " "	0	1	1	1	0	0	0	2	2	2	0	0
Glucose dehydrogenase + cozymase, cc	1	1	1	1	1	1	1	1	1	1	1	1
Methylene blue, cc	0	0	0	0	0	0.5	0	0	0	0	0	0.5
Buffer, cc	2	2	1	1	3	2.5	2	1*	0	0	3	2.5
2 M glucose, cc	0.15	0.15	0.15	0	0.15	0.15	0.15	0.15	0	0.15	0.15	0.15
Time, min	195	195	195	195	195	195	120	120	120	120	120	120
Oxygen uptake, c.mm	24.2	17.1	102.9	37.8	6.2	32.9	18.1	4.9	28.5	51.2	8.7	163

\* Water was used instead of buffer.

TABLE III  
*Adsorption of Cozymase by Indophenol Oxidase on Which Cytochrome Had Previously Been Adsorbed*

Vessel No.	Series A						Series B					
	1	2	3	4	5	6	1	2	3	4	5	6
Indo-cyto, cc	2	2	0	0	0	0	2	2	0	0	0	0
Indo-cyto-cozymase, cc	0	0	2	2	0	0	0	0	2	2	0	0
Supernatant fluid, cc	0.25	0.25	0	0	1	1	0.25	0.25	0	0	1	1
Glucose dehydrogenase, cc	1	1	1	1	1	1	1	1	1	1	1	1
2 M glucose, cc	0	0.15	0	0.15	0.15	0.15	0	0.15	0	0.15	0.15	0
Buffer, cc	1	1	1	1	2	1.5	2	1	1	1	2	2
Methylene blue, cc	0	0	0	0	0	0.5	0	0	0	0	0	0.5
Time, min	150	150	150	150	150	150	120	120	120	120	120	120
Oxygen uptake, c.mm	12.1	9.8	16.1	69.5	6.2	30.0	13.3	12.2	16.1	45.1	19.8	111.5

The data presented in Tables I to III are from the last twenty of about 100 experiments on the oxygen uptake of the systems described above.

*Inhibitory Effect of Fluid Remaining after Adsorption of Cytochrome on Permutit*—0.5 cc. of this fluid (characterized as "washing" in Table I) corresponds to 20 gm. of pressed yeast. The experiment was carried out in evacuated Thunberg tubes.

*Catalytic Effect of Cytochrome*—This was prepared from Fleischmann's yeast, according to the second method of Keilin as modified by Green and with additional adsorption on permutit and elution with ammonia solution. The final traces of silica were removed by treatment with 10 volumes of cold acetone in Series A of Table II, and by shaking with ether in Series B. The silica subsequently remained insoluble; the cytochrome dissolved in water. The cytochrome in each of Vessels 2, 3, and 4 of both series in Table II represents the yield from about 790 gm. of pressed yeast.

The differences between the oxygen uptakes of Vessels 5 and 6 of Series A in Table II and Vessels 5 and 6 of Series B are believed to be due to different lengths of exposure to atmospheric oxygen during solution on the dehydrogenase itself and on autoxidizable contaminants.

*Adsorption of Cozymase by Indophenol Oxidase on Which Cytochrome Had Previously Been Adsorbed*—The cytochrome used in forming the indo-cyto and indo-cyto-coenzyme employed in Vessels 1 to 4 of Series A in Table III represents the yield from 300 gm. of pressed yeast; that in Vessels 1 to 4 of Series B corresponds to 200 gm. of pressed yeast. The supernatant fluid in each case is that resulting from the centrifugation of portions of indo-cyto with cozymase solutions and the volumes added to the portions of indo-cyto not so treated are equal to the volume of the precipitate (indo-cyto-coenzyme) from which they have been separated.

#### SUMMARY

The phosphorus-containing fraction previously found associated with certain preparations of cytochrome C contains a substance inhibiting the action of the system; indophenol oxidase-cytochrome C-glucose-dehydrogenase-coenzyme-glucose.

It has been shown that the insoluble muscle substance (indo-

phenol oxidase preparation) adsorbs cytochrome C and cozymase, forming an active unit.

A new and convenient method for the preparation of active cytochrome C preparations from American yeast is described.

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# THE pH STABILITY OF THE PAPILLOMA VIRUS PROTEIN

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## PLATE 1

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In a previous note (1) we have described the ultracentrifugal isolation of a macromolecular protein from the warty tissue of western cottontail rabbits diseased with the virus of infectious papillomatosis (2). It was found that the infectivity of a tissue suspension was concentrated in the fraction containing this protein. The amount of protein that could be extracted from a tissue has been roughly proportional to its infectiousness; none has yet been obtained from the non-infectious warts that this virus induces in domestic rabbits. Such a close natural association of the infectivity and the protein indicates the importance of any experiments that allow us to compare their behaviors and stabilities under similar conditions. One of the most instructive experiments of this sort that can be made is a comparison of the pH ranges of virus activity and protein molecular stability. A preliminary account of such a comparison for the papilloma virus has already been published (3). Similar experiments have also been made with the elementary bodies of vaccinia (4) and the tobacco mosaic virus protein (5).

In the present paper are described the experiments which have served to define the pH limits within which the papilloma virus remains active and to measure the sedimentation rates and hence the approximate molecular weights of the purified heavy protein within and on either side of these pH limits. The virus stability was established by inoculating initially active virus kept for various periods of time at different hydrogen ion concentrations into the skin of susceptible rabbits and noting the number and size of the papillomas that developed after 4 weeks. The molecu-



lar constitution of the protein in these solutions was determined through ultracentrifugal analyses following the original absorption method of Svedberg.

The necessary purified protein was obtained in the manner previously outlined (1). The essential steps are the following. Glycerolated wart tissue of proved infectiousness was ground with sand and extracted with 10 times its weight of physiological saline. After clarification in a horizontal centrifuge, this extract was held for 1 hour in a centrifugal field (6) of about 60,000 times gravity. The infectious principle was concentrated in the solid pellets thus formed in the ultracentrifuge tubes. Supernatant liquids were accordingly discarded and the pellets resuspended in fresh saline. These suspensions were next freed of aggregated colloidal material in a Swedish angle centrifuge and the infectious solutions ultracentrifuged as before. This process of centrifugation and ultracentrifugation was repeated four or five times. By then the pellets were transparent and colorless; their concentrated solutions were equally colorless though strongly opalescent.

Experience showed that the original extracts contained light tissue proteins, the heavy protein with which the virus activity was associated, and much colloidal material of widely varying particle size. Most of the light protein was thrown away in the supernatant liquid from the first ultracentrifugation. That clinging to the solid pellets was eliminated more and more completely in the supernatant liquids of successive ultracentrifugal precipitations. Ordinarily no chemical test for protein could be obtained with supernatant liquids from the third or later sedimentations. The process of ultracentrifugal precipitation aggregated the colloidal material present so that more and more of it was removed by the low speed centrifugations that alternated with the ultracentrifugations.

The amount and homogeneity of the heavy protein obtained has been very different from different batches of warts. None was extracted from non-infectious tissue. Warts of low infectivity have given low yields and the protein from these "poor" warts has often produced in the analytical ultracentrifuge the diffuse boundaries indicative of molecular heterogeneity (Fig. 1); our very homogeneous preparations have all been derived from highly infectious tissue (Fig. 2). For the present experiments 18.2 mg.

of purified protein were employed. The wart tissue from which this was extracted weighed 105 gm., so that the averaged yield from the three bottles of warts supplying it was 1 part in about 5750.

For the study of the stability of infectiousness, papilloma virus was mixed with buffer solutions of desired pH. Portions of these solutions were tested for infectivity an hour, a day, a week, and 4 weeks after preparation by inoculation into shaved and scarified spots on the skin of domestic rabbits of the brown-gray, or agouti, breed (7). The resulting lesions were examined and charted three times each week. In some experiments the pH of the solution tested was readjusted to about pH 7.0 immediately prior to inoculation, while in others the mixtures were inoculated at the pH of the buffer-virus mixture. Throughout the work the recorded pH values are those of the virus mixtures as measured with a glass electrode. All virus solutions were kept until used in tightly stoppered Pyrex tubes immersed in ice water. These solutions were not sterile, but whenever practical they were handled with aseptic technique.

A composite buffer, based on that described by Best and Samuel (8), has been used in the studies of infectiousness. The stock solution contained boric acid, potassium dihydrogen phosphate, and potassium hydrogen phthalate each in 0.053 M concentration. This mixture exerts a satisfactory buffering action between pH 2 and 11, a range which proved to be adequate for the present work. To 50 cc. portions of the stock solution 0.2 M hydrochloric acid or sodium hydroxide was added to provide the desired pH, and the solution was then diluted to 160 cc. with distilled water. Buffer-virus mixtures were prepared by adding 2.0 cc. of solutions of the virus protein purified by ultracentrifugation to 8.0 cc. of the various dilute buffer solutions. The final concentration of the salt in the buffer-virus mixtures was thus 0.04 M.

In those experiments in which the pH was not readjusted, equal volumes of the buffer-virus solutions and the corresponding dilute buffers were mixed immediately prior to inoculation into the test rabbits. Control mixtures were prepared by adding virus to 0.9 or 0.3 per cent (0.05 M) saline without buffer salts. For those tests in which readjusted solutions were inoculated, a series of solutions was prepared by adding such an amount of 0.2 M hydro-

chloric acid or sodium hydroxide to 200 cc. of 0.05 M sodium chloride that a pH of 7.0 was attained when equal volumes of buffer-virus mixture and the appropriate readjustment solution were mixed.

Chart 1 shows the results of an experiment in which the pH values of the buffer-virus mixtures were readjusted to 7.0 before test inoculation; in the experiment of Chart 2 no such readjustment was made. It is apparent that the pH of the inoculum exerted no demonstrable influence on the infectivity of the virus. The rates of inactivation of the virus in the critical acid and alkali-

TESTS OF INFECTIVITY OF VIRUS KEPT AT DIFFERENT HYDROGEN ION CONCENTRATIONS WITH READJUSTMENT OF INOCULUM TO ABOUT PH7 PAPILLOMAS IN TEST RABBITS 30 DAYS AFTER INOCULATION

VIRUS PLUS	PH OF BUFFER BEFORE ADDING VIRUS	PH OF VIRUS MIXTURE AFTER 3 HOURS	TIME VIRUS WAS HELD AT DIFFERENT PH'S BEFORE INFECTIVITY TEST																PH OF VIRUS MIXTURE AFTER 4 WEEKS
			1 HOUR				24 HOURS				7 DAYS				4 WEEKS				
			RAB				RAB				RAB				RAB				
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
BUFFER	2.3	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.4	
"	3.2	3.2	++	++	+	+	+	+	+	0	0	0	0	0	0	0	0	3.2	
"	4.3	4.3	+++	++	+++	++	++	++	++	++	++	++	+++	+	++	++	++	4.3	
"	5.3	5.3	++	++	++	++	++	++	++	++	++	++	+	+	++	+	++	5.3	
"	6.4	6.4	++	++	++	++	++	+++	+++	++	++	++	++	+	++	++	++	6.4	
"	7.2	7.2	++	++	++	+	++	++	++	+	++	+	++	+	+	+	+	7.2	
"	8.3	8.3	++	++	+	+	++	++	+++	+	0	+	0	0	0	0	0	8.1	
"	9.2	9.2	+++	++	++	++	++	++	+	0	0	0	0	0	0	0	0	9.2	
"	10.3	10.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.5	
"	11.4	11.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.7	
SALINE 0.9 PER CENT	—	6.0	++	+++	++	++	++	+++	+++	++	+++	++	++	++	+	++	++	6.5	
SALINE 0.3 PER CENT	—	5.6	+++	+++	++	++	++	+++	+++	++	+++	+++	++	++	++	++	++	6.6	

0=NEGATIVE +1-5 SMALL PAPILLOMAS ++++=CONFLUENT PAPILLOMAS  
OTHER SYMBOLS SIGNIFY GROWTHS OF INTERMEDIATE SIZE.

CHART 1

line zones also were examined in more detail with a somewhat larger amount of the purified virus (Chart 3). It was clear from the lengthening of the period of incubation in test rabbits that no virus solution was quite as active at the end of 4 weeks as at the beginning of an experiment. The least loss of activity occurred in acid from neutrality to pH 4.2. At pH 3.0, inactivation was quite rapid and at pH 2.5 it was immediate. There was little indication that the virus was damaged more in buffer solution than in saline of the same pH. Initial activity was not long maintained in any alkaline solution. The virus instantaneously disappeared from solutions more alkaline than about pH 10. Less

alkaline solutions gradually lost infectivity, the rate of this inactivation increasing with the pH. It is interesting to note that

TESTS OF INECTIVITY OF VIRUS KEPT AT DIFFERENT HYDROGEN ION CONCENTRATIONS AND INOCULATED AT THE PH OF THE BUFFER-VIRUS MIXTURE WITHOUT READJUSTMENT PAPILLOMAS IN TEST RABBITS 30 DAYS AFTER INOCULATION

VIRUS PLUS	PH OF BUFFER BEFORE ADDING VIRUS	TIME VIRUS WAS HELD AT DIFFERENT PH'S BEFORE INECTIVITY TEST																PH OF BUFFER-VIRUS MIXTURE AFTER 30 DAYS
		1 HOUR				24 HOURS				7 DAYS				4 WEEKS				
		RAB				RAB				RAB				RAB				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
BUFFER	2.3	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.4
"	3.2	3.3	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	3.3
"	4.3	4.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	4.3
"	5.3	5.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	5.4
"	6.4	6.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	6.5
"	7.2	7.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7.3
"	8.3	8.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8.3
"	9.2	9.1	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	9.3
"	10.3	10.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.8
"	11.4	11.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.7
SALINE 0.9 PER CENT	-	6.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7.1
SALINE 0.3 PER CENT	-	6.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7.0

CHART 2

TESTS OF INECTIVITY OF VIRUS KEPT AT DIFFERENT HYDROGEN ION CONCENTRATIONS WITH READJUSTMENT OF INOCULUM TO ABOUT PH7. PAPILLOMAS IN TEST RABBITS 30 DAYS AFTER INOCULATION

VIRUS PLUS	PH BEFORE ADDING VIRUS	TIME VIRUS WAS HELD AT DIFFERENT PH'S BEFORE INECTIVITY TEST																							PH OF VIRUS MIXTURE AFTER 30 DAYS
		1 HOUR			6 HOURS			24 HOURS			48 HOURS			96 HOURS			8 DAYS								
		RAB			RAB			RAB			RAB			RAB			RAB								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
BUFFER	2.5	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5	
"	3.0	+	+	+	+	+	+	+	0	0	0	+	0	0	0	0	+	0	0	0	0	0	0	+	3.0
"	3.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3.5
"	4.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	4.0
"	4.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	4.5
"	7.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7.2
"	8.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7.9
"	8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8.2
"	9.0	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	8.4
"	9.5	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.8
"	10.0	+	+	+	0	+	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	9.0
SALINE 0.9 PER CENT	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

CHART 3

in acid, addition of the virus brought about little change in the pH of a buffer, while on the alkaline side its pH was sharply lowered.

The molecular state of the heavy protein in solutions between pH 1.5 and 11 has been determined by ultracentrifugal absorption analyses. Cells 2 mm. thick were employed; the absorption of the protein in the ultraviolet between 2300 and 2700 Å. is such that the optimum concentration for this thickness is about 1.8 mg. per cc. The solutions for analysis have in some instances been made by diluting a saline solution of twice the desired concentration of protein with an equal volume of 0.1 M buffer. In other cases a known amount of protein in saline solution was sedimented in the quantity ultracentrifuge and the resulting pellet dissolved in a measured amount of 0.05 M buffer. The accepted pH of the photographed solution was that measured on it with a glass electrode. The buffers were like those employed in the infectivity tests, except that potassium hydrogen phthalate was not used. This omission results in a poorer buffer, but it is necessary in view of the strong ultraviolet absorption of the phthalate ion.

The usual series of pictures showing the rate of protein sedimentation in a known centrifugal field were made immediately after preparing the solutions and after they had stood for various lengths of time. Sedimentation constants calculated from these photographs are collected in Table I; typical photographs are reproduced in Figs. 1 to 7. The protein has its isoelectric point at pH 5. Between pH 4.2 and 5.8, on either side of this point, it was too insoluble for measurement. It dissolved on the acid side with the same sedimentation constant that it had at pH 6. This value was maintained with increasing acidity until at a pH near to 2.8 the sedimentation rate suddenly dropped. This is undoubtedly due to a break in the protein molecule—light, “unsedimentable” material could be seen in considerable amounts in the diagrams of very acid solutions. The altered nature of the lighter acid protein was further demonstrated by its complete precipitation when the pH was readjusted to about 6.5.

The sedimentation diagrams from alkaline solutions were the same as that from the protein in neutral buffer until a pH slightly above 10 was reached. In solutions more alkaline than this the protein was split into fragments that were smaller the higher the pH (Table I). Such a stepwise splitting has already been described for the tobacco mosaic virus protein (5). It is to be

noted that both the acid and alkaline pH values at which there was molecular breakdown are, as closely as can be determined, those at which there is immediate loss of viral activity.

This parallelism between apparent molecular stability and infectiousness was not maintained in experiments on alkaline solutions that had stood for some time. As Charts 1 to 3 show, solu-

TABLE I  
*Sedimentation Constants of Papilloma Virus Protein*

pH	Sedimentation constant ( $s_{20}^0$ )
1.5	Too small to measure
1.85	182, 297
2.6	203, 302; 214, 320 (30 days)*
2.7	202
2.85	194
2.9†	276, 408; 278, 424 (39 days)
3.3	263, 392
4.0	269
Insoluble	
6.0	267, 381
6.5	264, 385
Saline	284, 414; 283, 426 (36 days)
7.0	251, 378
9.5	254, 374
9.9	251, 373
10.2†	129
10.7	30

\* Numbers in parentheses indicate the length of time in days that the preparation has been at the designated pH before measurement.

† The virus is immediately inactivated at pH values lower than about 2.9 and higher than about 10.1.

tions in the pH range between 7 and 10 became non-infectious at a rate that increased with the alkalinity. We have not been able to demonstrate a change in sedimentation diagrams accompanying these slow losses of infectivity. Weakly alkaline solutions of the tobacco mosaic virus protein behave in the same way (5, 8). If infectiousness is a property of the heavy protein, this must mean that slow alkaline inactivation is due to chemical changes that

do not effect a measurable alteration in the molecular size and shape. The sedimentation constants for the molecules in the unstable but still active solutions having pH values between 7 and 10 appear to be a few per cent lower than for the molecules in the more stable solutions at pH 6 and 6.5 (Table I); they are definitely smaller than the constants for solutions in physiological saline. The real meaning of these small differences in sedimentation rate is not clear. They could arise through minor changes in molecular shape or size, or they could conceivably have their origin less directly in phenomena associated with the known great hydration of these molecules.

The molecular weight ( $M$ ) of the heavy protein cannot be established without a knowledge of its density and of its diffusion rate. Neither has yet been measured. If the density is  $\rho =$  about 1.33, as in the case of most proteins, and if the diffusion constant were to be near  $D = 1.0 \times 10^{-7}$  (as could reasonably be true) (9), then  $M$  would be around 25 millions.

Two sedimenting boundaries can be seen in the ultracentrifugal diagrams of the purified protein (Fig. 5). The nature of the heavier secondary boundary is uncertain. It is a common feature in diagrams of the heavier proteins, especially those that have been subjected to the action of salts (10). It might be assumed that solutions giving these double boundaries contained 2 unrelated molecules that happened to have nearly the same weights. There are, however, several facts that argue against such a conclusion. The absorption of light responsible for the secondary boundary, for example, often increases as a given solution ages. Other evidence that the two boundaries are related is to be found in the fact that originally double boundaried preparations of the papilloma protein usually show only the principal boundary when they go back into solution on the acid side of the isoelectric point. Svedberg (9) has found that molecular association can account for somewhat similar double boundaries among the lighter proteins with which he has dealt. The sedimentation constants of the two boundaries of the papilloma protein are different enough to be an expression of such association, but the constants observed for other virus proteins could not be explained in this way.

## SUMMARY

A study has been made of the pH range of stability of the molecules and the infectiousness of solutions of the purified heavy protein isolated by quantity ultracentrifugation from virus-induced warts on cottontail rabbits. It is found that immediate inactivation of the virus occurs at those pH values at which the protein molecules fragment. In weakly alkaline solutions there is a gradual loss of viral activity which is not reflected in observable changes in the molecular sedimentation rates. The observed phenomena are compatible with the hypothesis that the homogeneous heavy protein is the causative agent of infectious papillomatosis in rabbits.

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## EXPLANATION OF PLATE 1

FIG. 1. The sedimentation diagram of the inhomogeneous protein extracted from several "poor," only weakly infectious warts. Mean centrifugal field = 10,600 times gravity. In Figs. 1 to 7 the interval between successive pictures was exactly 5 minutes; the duration of an exposure was about 1.5 seconds. Photography was with ultraviolet light of wave-lengths between about 2300 Å. and about 2700 Å.

FIG. 2. The sedimentation diagram of a saline solution of homogeneous protein from highly infectious warts. No change could be seen in a photograph made after the solution had stood for 36 days in the ice box. Note the fainter and more rapidly sedimenting secondary boundary. Mean field = 16,600 times gravity.

FIG. 3. The diagram of an inactive pH 1.8 solution of the papilloma protein. Though it sedimented at a slower rate (Table I) than the active



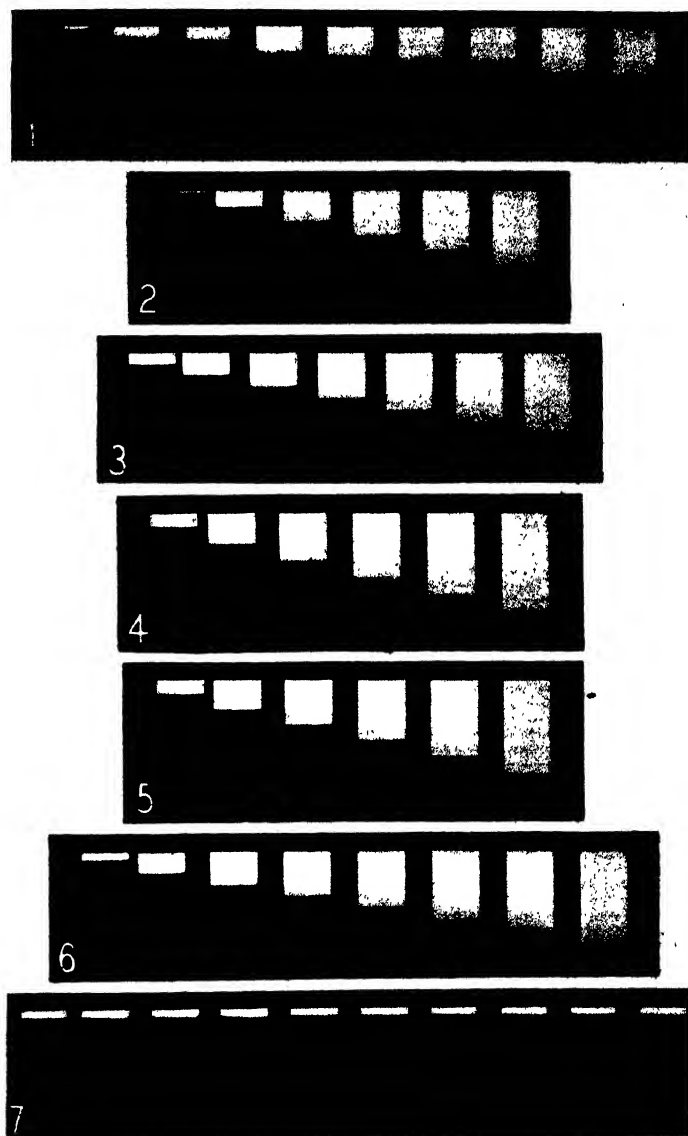
proteins, it had the same homogeneity. Unsedimentable material can be seen in the original negative. Mean field = 16,710 times gravity.

FIG. 4. Diagram of a solution of the papilloma protein in pH 2.9 buffer. The sedimentation rate deduced from this photograph is the same as that found for solutions on the alkaline side of the isoelectric point. Mean field = 16,810 times gravity.

FIG. 5. Diagram of the papilloma protein in pH 7.0 buffer. Mean field = 17,380 times gravity.

FIG. 6. Diagram of the papilloma protein in pH 9.6 buffer. There is no measurable difference in the diagrams of an active solution immediately after adjustment to this pH and of the same solution after it had lost activity on standing. Mean field = 12,830 times gravity.

FIG. 7. Diagram of an inactive solution of pH 10.7. The molecular fragments are small but homogeneous in size; much "unsedimentable" material is also present. Mean field = 10,510 times gravity.



(Beard and Wyckoff: pH of papilloma virus protein)



# THE BIOLOGICAL ASSAY OF THE CARBOHYDRATE METABOLISM HORMONE\* OF THE ANTERIOR PITUITARY†

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WITH THE TECHNICAL ASSISTANCE OF P. T. CUPPS

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Since Johns *et al.* (1) produced hyperglycemia and glycosuria in normal dogs by the injection of extracts of the anterior pituitary, and Koster and Geesink (2) observed subnormal values for blood sugar in hypophysectomized dogs, there has been increasing evidence presented for a "carbohydrate metabolism" hormone secreted by the anterior pituitary (see reviews by van Dyke (3), and Gomez and Turner (4)). The importance of this factor in lactating animals was indicated by the work of Gomez and Turner (5) who observed that lactation, in hypophysectomized guinea pigs, could be initiated or maintained with the lactogenic and adrenotropic hormones only when frequent injections of glucose were made, and by the observations of Graham (6) concerning the precursors of lactose and the relation of the latter to the control of the volume of milk secreted. As the secretion of large amounts of milk requires the mobilization of large amounts of blood carbohydrates, it was decided to begin a study of the carbohydrate metabolism hormone of the anterior pituitary.

The first effort was directed toward the development of a satisfactory biological assay method. Eitel and Loeser (7) and Holden (8) showed that pituitary extracts increased the blood

\* Has been called diabetogenic, contrainsulin, blood sugar-raising principle, glycotropic, and glycogenolytic factor.

† Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series, No. 549.

sugar and decreased the liver glycogen in immature guinea pigs (200 gm. or less). Holden determined the blood sugar of guinea pigs 24 hours after a series of injections of an acid extract of cattle pituitary. The results were not consistent, ranging from 92 to 384 mg. per cent. In one-third to one-half of the determinations, values were obtained exceeding the highest normal levels. In order to find the maximum level, he determined the blood sugar after single injections as follows: after 4 hours, 170 mg. per cent; after 8 hours, 232 mg. per cent; after 13 hours, 204 mg. per cent; and after 24 hours, 138 mg. per cent.

Preliminary studies were designed to verify Holden's observations as to the interval following the injection when a maximum response would be obtained, to determine the effect of varying dosage, and to determine the variability of response between different animals.

Very soon it was discovered that the nutritive condition of the test animals greatly influenced the sensitivity of the response to the hormone. It should be emphasized that only animals which are eating well and gaining in weight normally should be selected for the test. In animals not in good condition and not eating well the liver glycogen was found to range between 252 and 768 mg. per cent as compared to a normal of about 2500 mg. per cent.

To obtain a rapid increase in the blood sugar it appeared desirable for the rate of absorption to be as rapid as possible. Since it is generally recognized that the intraperitoneal method of injection permits more rapid absorption than the subcutaneous route, the former was used.

*Normal Blood Sugar Values for the Guinea Pig*—The normal range of blood sugar in the guinea pig reported by Eitel and Loeser (7) was 68 to 120 mg. per cent, determined by the Hagedorn-Jensen method. Holden (8) used the Shaffer-Somogyi method and reported a range of 90 to 126 mg. per cent in males weighing 190 to 200 gm.

In twenty-three normal male guinea pigs within a weight range of 147 to 220 gm., the blood sugar determined by the Shaffer-Somogyi method ranged between 90 and 126 mg. per cent with an average of 108.6 mg. per cent. In this group are included six animals which received 5 mg. of an extract of beef muscle. A group of eight animals ranging in weight from 257 to 375 gm.

ranged from 99 to 134 mg. per cent with an average of 115.1 mg. per cent (Table I). These and other data lead us to believe that body weight does not markedly influence the blood sugar. We prefer, however, to use animals within the weight range of about 180 to 220 gm., so that variations due to the effect of body weight

TABLE I  
*Relation between Blood Sugar and Body Weight of Normal Guinea Pigs*

Body weight	Blood sugar	Body weight	Blood sugar
gm.	mg. per cent	gm.	mg. per cent
147*	107	257	116
150*	108	257	134
157*	105	270	105
158	107	270	107
160*	104	297	130
163	105	290*	117
164	105	316	113
165	107	375	99
166	105		
168	90		
170	90		
175	114		
178	115		
180	107		
180	122		
180	105		
183	112		
185	126		
185	122		
195	104		
200	110		
215	113		
220*	115		
Average..175.8	108.6	291.5	115.1

\* Injected with 5 mg. of beef muscle extract.

in relation to the amount of hormone injected may be kept at a minimum.

#### EXPERIMENTAL

*Procedure*—Guinea pigs weighing from 180 to 220 gm. in good physical condition, eating normally and gaining in weight, were

selected. Intraperitoneal injections were usually made at 8 a.m. The animals were then sacrificed at varying intervals by a sharp blow on the head. The blood sample was obtained by severing the blood vessels of the neck. Blood sugar determinations were then made by the Shaffer-Somogyi (9) method. The animals were handled as little as possible previous to the time of sacrifice to eliminate blood sugar elevation due to fright or other psychic causes.

Except as otherwise noted, the hormone used in all of the experiments reported in this paper was extracted from acetone-dried sheep anterior lobe powder with 15 to 20 volumes of 60 per cent alcohol at a pH of 9 to 10. The supernatant fluid from three successive extractions was combined, and the active principles precipitated by adjusting the solution to a pH of about 5.7 and by the addition of 95 per cent alcohol (about 3 volumes) until the concentration of alcohol was 86 per cent or above. The precipitate which formed was removed with a centrifuge, washed four times with 95 per cent alcohol and twice with ether, then dried in a current of warm air.

For the purpose of injection, the dried preparation was dissolved in a slightly alkaline solution (with NaOH), then precipitated by bringing the solution to the isoelectric point with HCl.

*Observations*—In the preliminary experiment a total of forty-seven guinea pigs was injected with amounts of extract varying from 3 to 20 mg. and then sacrificed at intervals varying from 4 to 28 hours (Table II). Considerable variation was observed in the blood sugar level, ranging from 97 to 309 mg. per cent. Even under the same conditions some animals failed to respond, remaining within the limits of the normal range. Upon seeking a possible explanation for these low values, it was noted that in most cases such animals either lost or failed to gain in weight during the day of the injection. It appears important that the food intake on the day of injection be adequate to provide an abundant absorption of carbohydrates.

Very definite increases in blood sugar were induced with injections of 4 or more mg. of the extract, thus confirming the observations of Holden and demonstrating the potency of our preparations. A period of 8 hours between the time of injection and the

sacrifice of the animal resulted in satisfactory elevations of the blood sugar. With the higher levels of hormone injection, sugar levels of 200 mg. per cent or above were noted. Considering that the excess sugar would appear in the urine rather rapidly, it is not surprising that the larger amounts of hormone (10 and 20 mg.) did not increase the sugar much above this level, but produced a more sustained effect.

TABLE II  
*Effect of Pituitary Extract upon Blood Sugar (Body Weight between 180 and 220 Gm.)*

Interval between injection and sacrifice	Blood sugar following anterior pituitary extract					
	3.0 mg.	3.5 mg.	4.0 mg.	5.0 mg.	10.0 mg.	20.0 mg.
hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
4				175	183	172
6	136, 116	127, 121	207, 168, <b>112</b> , <b>116</b>			
8	138, 136, 111, 128, <b>108</b> , <b>107</b> , average 121.3	142, <b>115</b>	180, 206, 169, 168, 202, <b>126</b> , <b>124</b> , average 167.9	206, 216, 187, average 203.0	309	232
10		137, <b>97</b>	158, 211			
12		149, <b>116</b>	132, 195	171	<b>110</b>	242
14				182		
16					145	220
28					133	179

The bold-faced figures represent blood values from animals which either lost or failed to gain weight.

*Guinea Pig Unit of Carbohydrate Metabolism Hormone*—On the basis of the observations in Table II, it was decided to formulate a tentative procedure for the biological assay of this factor. Male guinea pigs weighing 180 to 220 gm. are to be used. The pituitary extract, in the form of a suspension, is injected intraperitoneally in the morning and the animals sacrificed 8 hours later. The blood sugar is determined by the Shaffer-Somogyi (9) method. 1 unit of hormone is defined as that minimum amount which will increase the blood sugar of at least five animals



under these conditions about 50 per cent above the normal (normal about 110) or to about 165 mg. per cent.

As 4 mg. of hormone produced an average blood sugar of 168 mg. per cent (see Table II) in the preliminary trial, it was decided to determine the reproducibility of the results under uniform conditions at the same level of injection, with the following results.

	<i>mg. per cent</i>	<i>Average mg. per cent</i>
Group II.....	195, 157, 156, 170, 141	163.8 (5 animals)
“ III.....	175, 140, 221, 181	179.3 (4 “ )
“ IV.....	147, 100, 144, 170, 99	132.0 (5 “ )
“ V.....	140, 164, 151, 165, 208, 142	161.7 (6 “ )
“ VI.....	156, 133, 213, 167, 136	161.0 (5 “ )

The average of thirty-two animals was 160.8 mg. per cent of glucose.

Of the six groups of animals each injected with 4 mg. of the initial extract of the anterior pituitary, four groups averaged between 160 and 170 mg. per cent, while one was slightly high and the other low, due to the presence of two animals within the normal range. Of thirty-two animals included in the entire series of assays, twenty-eight were above the normal range. It is believed that by the careful selection of animals in a thriving condition, the number of non-responsive animals can be kept at a minimum. Five animals will usually give a good preliminary assay, and may be relied upon if all individuals give a response above the normal range. If there are individuals within the normal range, the number of animals should be increased to ten or more.

Cattle pituitary glands extracted by the procedure outlined above were also assayed by this method for their content of the carbohydrate metabolism factor. An injection of 4 mg. of the extract produced the following elevations in the blood sugar of the test animals: 172, 230, 136, 166, 195, 175, 133, 167, and 158, with an average for the nine of 170.2 mg. per cent. It would thus appear that these samples of sheep and cattle pituitaries contain about the same amount of the hormone.

#### SUMMARY

A study is reported on the use of the guinea pig in the biological assay of the carbohydrate metabolism hormone of the anterior

pituitary. The blood sugar of the normal male guinea pig within the weight range of between 150 and 220 gm. varied between 90 and 126 mg. per cent as determined by the Shaffer-Somogyi method, with the average of twenty-three such animals 108.6 mg. per cent.

It was found possible to confirm the observations of earlier workers indicating the presence in pituitary extracts of a factor which would cause a rapid elevation in the level of the blood glucose of well nourished guinea pigs.

In preliminary studies a total of forty-seven guinea pigs was injected with varying amounts of the pituitary and then sacrificed at intervals varying from 4 to 28 hours. Increases in blood sugar approaching the maximum were attained in about 8 hours.

Upon these observations, a tentative guinea pig unit of the hormone was formulated as follows: a unit of the carbohydrate metabolism hormone is defined as the minimum amount of extract injected intraperitoneally into well nourished male guinea pigs weighing from 180 to 220 gm., which will cause after 8 hours an average increase of 50 per cent (to about 165 mg. per cent) in the blood sugar of five or more animals.

Five groups of animals showed that the reproducibility of the method was good. The selection of well nourished animals is of prime importance.

Samples of sheep and cattle pituitary extracted in 60 per cent alcohol at a pH of 9 to 10 contained about 250 units per gm. of dried extract (4 mg. per unit).

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## A STUDY OF THE INFLUENCE OF HEAVY WATER UPON AMYLASE FORMATION IN BARLEY

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Amylase activity increases markedly during the germination of many grains and this increase is often associated with the formation or liberation of more than one starch-splitting enzyme. Thus, malted barley has been found (1-7) to contain at least two distinct amylases in proportions which depend upon the treatment of the grain. Water is an important factor in these changes and is also essential to the action of the amylases after they are formed. It seemed of special interest, therefore, to study the influence of heavy<sup>1</sup> as compared with ordinary<sup>2</sup> water upon the generation of amylase activities during the sprouting of barley. The results of this extensive quantitative study are briefly summarized here.

The study included a comparison of the amylase activities of barley grains before germination with those of grains which had been allowed to germinate in water<sup>2</sup> and in different concentrations (8) of heavy water. The comparisons were made at definite intervals during the course of the germination and also at what appeared to be the same stages of germination of the grains. The concentrations of heavy water studied were 1, 10, 50, and 100 per cent (8). Precautions already described (8) were observed to prevent as far as possible dilution of the heavy water by water other than that present in the air-dry grains.

### EXPERIMENTAL

*Enzyme Solutions*—The enzyme solutions were prepared in a strictly uniform manner. The barley was soaked for 3 hours (9)

<sup>1</sup> The heavy water was kindly furnished by Professor H. C. Urey.

<sup>2</sup> All of the ordinary water as well as the heavy water used in this work was especially purified as previously described (8).

in the concentration of water being studied. Equal numbers of grains from each comparison were then placed, cleft side down, upon a layer of small glass beads (1 mm.) (9) in Petri dishes and moistened with equal volumes of the water being studied in each case. The dishes were placed in desiccators over water of the same concentration. The desiccators were kept side by side at room temperature (23-29°) in a darkened room.

In one type of comparison, equal numbers of grains were allowed to germinate for the same lengths of time in the different concentrations of water. They were then lifted from the beads, ground in a glass mortar, extracted with equal volumes of ordinary<sup>2</sup> water (usually 5 cc.), centrifuged, and the supernatant liquid examined for amylase action. The time of extraction and that of centrifuging were kept constant.

In the second type of experiment, equal numbers of grains which had reached what appeared to be the same stage of germination were selected from each comparison, treated as above, and examined for amylase action.

*Measurements of Amylase Activity*-- Measured portions of the extracts obtained as described above were allowed to react for definite time intervals at 40° with suitably buffered (6) dispersions of soluble potato starch made up in purified ordinary water. The reducing sugar formed from the starch was measured iodometrically (10). The starch-splitting activity was determined by measurements of the velocity of the disappearance from the hydrolysis mixtures of products which give a blue color with iodine (11) and, in some cases, by measurements of the residual starch or amylose at intervals during its hydrolysis (12).

### *Results*

Suitably controlled experiments showed that heavy water, in concentrations equivalent to those introduced into the hydrolysis mixtures by the enzyme solutions did not interfere with the hydrolysis of the amylose by the amylases under the conditions of these experiments. This made it possible to make strictly comparable measurements of the amylase action of the different extracts studied.

Both the saccharogenic and the amyloclastic activities of the extracts were found to increase with the time of germination of the

grain in all the systems studied, but the increases in the two types of amylase action were not proportional in any case; the amylolytic activity, which is negligible in the ungerminated barley, rose much more rapidly in all cases than the saccharogenic activity which is already relatively high in the dormant barley.

TABLE I  
*Influence of Heavy Water upon Amylase Formation in Barley*

Treatment of barley	Average length of roots	Saccharogenic action		Amylolytic action	
		Activity*	Relative increase	Activity†	Relative increase
	<i>mm.</i>				
Ungerminated.....		297	1.0	<2	1
After 24 hrs. in H <sub>2</sub> O.....	4	595	2.0	100	50
“ “ “ “ 10% D <sub>2</sub> O.....	4	595	2.0	100	50
“ “ “ “ 50% “.....	2	379	2.1	100	50
“ “ “ “ 100% “.....	?	305	1.0	<8	5
“ 48 “ “ H <sub>2</sub> O.....	19	1086	3.7	871	436
“ “ “ “ 10% D <sub>2</sub> O.....	19	1086	3.7	871	436
“ “ “ “ 50% “.....	11	1009	3.4	555	278
“ “ “ “ 100% “.....	1	518	1.7	33	17
“ 72 “ “ H <sub>2</sub> O.....	40	1817	6.1	1714	857
“ “ “ “ 10% D <sub>2</sub> O.....	40	1817	6.1	1714	857
“ “ “ “ 50% “.....	12	1353	4.6	1000	500
“ “ “ “ 100% “.....	1.5	505	1.7	50	25
“ 120 “ “ H <sub>2</sub> O.....	50	1549	5.2	1818	909
“ “ “ “ 50% D <sub>2</sub> O.....	20	1549	5.2	1429	715
“ “ “ “ 100% “.....	2.0	834	2.8	63	31

\* The saccharogenic activity was calculated from the volumes of the enzyme solutions required to give 15 mg. of maltose per 5 cc. of 2 per cent starch in 30 minutes at 40° by dividing the weight of maltose in mg. by the volume in cc. of the enzyme solution.

† The amylolytic activity was calculated from the volumes of the enzyme solutions required to bring 5 cc. of 1 per cent starch in 30 minutes at 40° to products which give a clear red color with iodine, by dividing the weight of the starch (50 mg.) by the volume in cc. of the enzyme solution.

This lack of proportionality in the two activities is in accord with, and lends further support to, other evidence (1-7) that more than one amylase is generated during the germination and sprouting of barley.

The extent and rate of sprouting of barley and the generation

of amylase activities were not appreciably different in grains which had been treated with ordinary water or with 1 per cent or 10 per cent heavy water. These observations, repeatedly made with several lots of barley, are of interest as an indication that heavy water is not appreciably unfavorable to the metabolism of this grain even when present in concentrations many times higher than those which are probably normally encountered in nature.

100 per cent heavy water, however, had a markedly unfavorable influence upon the rate and extent of the sprouting of the barley and upon the generation of the amylase activities which is affected in the same direction as the germination of the grain. Moreover, the unfavorable influence of the higher concentrations of heavy water was much more pronounced with the starch-splitting than with the sugar-forming activity. Typical, comparable data are given in Table I.

As the heavy water was found not to influence appreciably the activities of these amylases in the crude extracts here studied, its unfavorable influence would seem to be exerted either upon the stabilities of these amylases, especially that of the amylase with relatively high starch-splitting activity ( $\alpha$ -), upon the stabilities of inactive combinations of these amylases in the grain, or upon other enzymes in the grain which are normally needed to set free or to form the amylases from some inactive combination or state. The first of these possibilities is being studied with highly purified amylase preparations from barley and from malted barley.

#### SUMMARY

Heavy water, in concentrations of 1 and of 10 per cent, has no appreciable influence upon the germination of barley or upon the generation of amylases during this process. 100 per cent heavy water, however, has a markedly unfavorable influence upon the rate and extent of the sprouting of barley and upon the generation of amylase activities, and this unfavorable influence is much more pronounced with the amylase of relatively high amylolytic ( $\alpha$ -amylase) than with the amylase of relatively high sugar-forming activity ( $\beta$ -amylase).

The unfavorable influence of the heavy water appears to be exerted upon the formation or upon the stabilities of the amylases rather than upon their action in the hydrolysis of starch.

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# THE DIFFERENTIAL MIGRATION OF THE PRESSOR AND OXYTOCIC HORMONES IN ELECTROPHORETIC STUDIES OF THE UNTREATED PRESS-JUICE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND

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A study of the electrophoretic behavior of preparations obtained by chemical procedures from the posterior lobe of the pituitary gland has shown that both the pressor and oxytocic activities migrate toward the cathode, the pressor migrating at the faster rate (1). The fact that the two activities possess different migration rates makes it obvious that, in such preparations, these activities are properties of separate entities. This is in agreement with the preponderance of evidence available at the present time. On the other hand, one cannot assume from these data that the activities were originally present as separate entities, since the methods of preparation of the active powders involved rather drastic treatment. It is entirely possible, as has been claimed, that these activities are actually manifestations of a single compound which is split at some stage in the chemical purification process into separate pressor and oxytocic components.

It occurred to us that if we could in some way study the electrophoretic behavior of fresh gland material which had been subjected to no chemical treatments whatsoever, we might secure crucial evidence on this point. It was therefore decided to study the electrophoretic migration rates of the pressor and oxytocic activities from the mechanically expressed juice of fresh untreated glands.

The first experiments were carried out on posterior lobes which

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were frozen in dry ice and sent to us from Detroit.<sup>1</sup> The glands were thawed, ground with sand, and immediately subjected to 20,000 pounds pressure in a Carver press. The temperature during the pressing operation was maintained below 10°. The pressor and oxytocic activity contained in the juice and in several washes obtained by this procedure was compared with that obtainable from an equal weight of glands by the usual hot 0.25 per cent acetic acid extraction of the acetone-desiccated material. Interestingly enough, within the accuracy of the methods of assay, practically all of the pressor and oxytocic activity extractable from the glands was obtained in the press-juice and the wash of the sand-gland residue. This active press-juice was placed directly in the electrophoresis apparatus and the relative rates of migration of the pressor and oxytocic activities were determined by assay. As was found in the electrophoresis of chemically derived crude powders, the pressor activity from the press-juice migrated more rapidly than the oxytocic. This observation was confirmed repeatedly, when the press-juice obtained from several batches of frozen glands was used.

Despite the fact that no chemical treatments were employed in preparing the material for electrophoresis, we felt that the results were open to some question, inasmuch as we did not have complete data on the previous history of the glands used. Enzymatic changes might have taken place in the gland material prior to freezing or during the period of storage and shipment in the frozen condition. For this reason, we next studied the freshest gland material obtainable, under conditions controlled so as to minimize possible enzymatic action. Arrangements were therefore made to obtain posterior lobes from cattle within a few minutes after the death of the animal on the killing floor of the slaughter-house. The hypophysis was removed and the posterior lobe immediately dissected out and frozen in dry ice. The lobes were then brought as quickly as possible to the laboratory where they were thawed momentarily for the expression of the active juice. The juice was then subjected to electrophoresis at a temperature maintained below 10°. Assays made after the completion of the electro-

<sup>1</sup> We wish to express our appreciation to Dr. Oliver Kamm of Parke, Davis and Company for so generously supplying us with frozen gland material.

phoresis again showed that the pressor activity migrated at a greater rate than the oxytocic.

Since the two activities migrate at different rates they must obviously be manifestations of separate chemical entities in this mechanically expressed juice obtained from fresh glands. This, of course, cannot be taken to indicate that the same situation necessarily exists in the gland itself. The elimination of such a possibility is beyond the scope of the present approach.

It is quite apparent that this same method of approach which we have found effective in the case of the posterior pituitary might well be applicable to other problems where the question arises as to whether or not various activities are due to a single substance or to different substances. The problem confronting the field with regard to the physiological activities of the anterior lobe of the pituitary gland immediately comes to mind as an instance in which this approach might be particularly effective.

#### EXPERIMENTAL

The apparatus used in this investigation has been described in detail in the previous paper (1). The electrophoresis set-up used consisted of a 5 cell series of 30 cc. beakers. The electrodes were of pure carbon. 1000 volts direct current were used throughout the work.

A typical experiment on the electrophoresis of the press-juice from fresh glands will be presented in detail. This experiment was confirmed by three separate experiments in which fresh gland material was collected and subjected to the same procedure described in the typical experiment. The results were in complete agreement with those of the experiment to be described.

Thirty-four posterior lobes, representing 10 gm. of material, were dissected from the hypophyses of steers within a few minutes after the killing of the animals.<sup>2</sup> The lobes were rinsed with water to remove excess blood and were immediately frozen by dropping them into a test-tube immersed in dry ice. The glands, brought to the laboratory in the frozen state, were thawed sufficiently to permit them to be quickly and thoroughly ground in a chilled porcelain mortar with 10 gm. of clean white sand. The juice was

<sup>2</sup> We wish to thank Greenwald, Inc., Baltimore, for making available to us the fresh gland material used in this work.

expressed from the ground mass by means of 20,000 pounds pressure in a Carver press. All of the parts of the press which touched the material during the pressing operation were previously chilled. A few cc. of ice water were added to the residue. The material was again ground and was subjected to pressure. This process was again repeated. The juice and the washings were combined and diluted to 100 cc. with ice-cold distilled water. The pH of this solution was 6.0. 20 cc. of this diluted juice, representing 2 gm. of glandular material and containing 560 units of pressor and 420 units of oxytocic activity, were placed in the center cell of the 5 cell set-up. The remaining cells were filled with 20 cc. of distilled water; the siphons were filled by application of suction to the manifold. The entire set-up was maintained at a temperature

TABLE I  
*Distribution of Activities after Electrophoresis. Experiment 1*

Cell No.	Pressor units	Oxytocic units	pH
1 (Cathode)	27	3	10.2
2	432	240	6.2
3 (Starting)	96	170	4.0
4	3	9	3.0
5 (Anode)	0	0	2.3
Totals . . . . .	558	422	

below 10° by immersing it in an ice-cooled water bath. Electrophoresis at 1000 volts was carried out for 17 hours, after which the cell contents were removed and assayed. The current, during the experiment, started at 0.17 milliamperes, increased rapidly to a peak of 4.28 milliamperes at the end of 6 hours, and had decreased gradually to 0.49 milliamperes by the end of the run. The time elapsing from the removal of the glands from the animals to the end of the electrophoresis was 23 hours, during which period the temperature of the glands and the gland juice was never above 10°.

The results of the assays, as well as the pH values of the cells at the end of the period of electrophoresis, are given in Table I. The pressor assays were carried out on cats under deep amytal anesthesia, and oxytocic assays were made on hens, with the

technique described in previous work reported from this laboratory (2).

The results given in Table I show definitely that the pressor and oxytocic activities contained in the fresh gland juice migrate at different rates toward the cathode cell, the pressor migrating at the greater speed. The ratio of pressor to oxytocic activity in the cathode cell after completion of the electrophoresis was 9:1. The same ratio in the material placed in Cell 3 at the start of the electrophoresis was 1.3:1. It was clear that the differential migration was not due to preferential inactivation, since the total amount of both activities started with was accounted for at the end of the electrophoretic period. The difference in the relative migration rates of the two activities is further demonstrated by the assay values given for Cells 2 and 3. There was relatively more pressor than oxytocic activity in Cell 2, whereas in Cell 3 the reverse was true.

#### SUMMARY

In the electrophoretic study of the chemically untreated, mechanically expressed juice of fresh posterior pituitary glands, it was found that the pressor activity traveled at a faster rate than the oxytocic activity, thus demonstrating that the activities in this simple press-juice were manifestations of different chemical entities.

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# THE FORMATION OF COPROPORPHYRIN I AND HEMOGLOBIN DURING EMBRYONIC LIFE

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The experiments reported in this communication concern the qualitative and quantitative measurement of the amount of coproporphyrin I and hemoglobin formed (as a measure of the Type III porphyrin) at intervals during the development of the chick embryo.

The coexistence of Type I and Type III porphyrins has been shown in yeast (1), and in human beings (2-7) as well as in animals (8, 9). Fischer has discussed this fact (10), and has termed it "the dualism of the porphyrins." Coproporphyrin I is produced and excreted under normal and most pathological conditions (7). The substance seems to play no functional rôle in the body, however, since the respiratory pigments hemoglobin, myoglobin, cytochrome C, and catalase are all Type III porphyrin compounds. Fischer has certain experimental evidence which supports the view that Type I porphyrin is synthesized independently of Type III porphyrin (10, 11). He considers the production of the Type I compound to be an atavistic phenomenon (11). This view is supported by Borst and Königsdörffer (12). Duesberg (13) believes that the coproporphyrin excreted in pernicious anemia is formed by a pathological activity of the bone marrow, whereas Schreus (14) believes that the coproporphyrin excreted in certain pathological conditions is the product of the action of intestinal bacteria.

Dobriner and coworkers (4-8) have presented clinical and experimental studies which indicate that coproporphyrin I is a by-product of the synthesis of the Type III porphyrins, which in turn form the respiratory pigments. Their evidence indicates that



normally a constant ratio exists between the rates of production of the two types of compounds. Their conclusions are in accord with the results of Rimington's (9) studies on congenital bovine porphyria. All the experiments from which their conclusions have been drawn as to the proportional formation of Type I and Type III depend upon simultaneous changes in the rate of formation of both substances with induced changes in the rate of hematopoiesis.

In order to study the biological relationship between Type I and Type III porphyrins, qualitative and quantitative tests for coproporphyrin have been made during the early phases of development of the chick embryo in order to ascertain whether coproporphyrin I is present and if so whether it appears with the onset of hematopoietic activity. Furthermore, a study has been made as to whether a constant proportion exists between the amounts of Type I and Type III porphyrins produced.

Relatively few studies of porphyrin formation in embryonic life have been made. Borst and Königsdörffer (12) have demonstrated, by methods depending upon fluorescence, that coproporphyrin is present in the bones of the human embryo of 4 months. Günther (15) found coproporphyrin in the meconium of fetuses of 4 to 6 months and Stockvis (16) found coproporphyrin in large amounts in the meconium after birth. Waldenström (17) identified the coproporphyrin in the meconium as Type I. Fränkel (18) and Borst and Königsdörffer (12) state that the placenta is impermeable to porphyrins, and hence assume that the coproporphyrin found in the embryo is an endogenous product. Fikentscher (19) states that the serum of a human fetus of 4 to 6 months of age contains 8 to 10 micrograms of coproporphyrin per 100 cc. The same author found coproporphyrin in amniotic fluid (20).

Fischer and his coworkers (21, 22) showed that protoporphyrin Type III is present in the egg-shells of certain species. Klose and Almquist (23) showed that protoporphyrin is contained in the vitellin membrane of hen's eggs. Van den Bergh and Grotepass have reported the presence of protoporphyrin in incubated eggs (24). No studies are available, however, concerning the presence of coproporphyrin in the hen's egg.

By employing the closed system of the egg any question as to an

exogenous source of coproporphyrin is eliminated, since experiments showed no detectable amount of that compound in the shell. The conversion of Type III porphyrin, resulting from the breakdown of erythrocytes, into the Type I compound was rendered unlikely by studying the period of embryonic development before any destruction of erythrocytes occurs, as shown by the absence of bile pigment. Sendju (25) found no bile pigment in chick embryos of 7 days incubation, whereas 0.2 mg. was present after 14 days.

### *Methods*

Over 600 eggs were used in the experiment. They were incubated at 38° in an ordinary bacteriological incubator. The shells were removed and used for measurement of the content of coproporphyrin I. The entire embryos were then removed and pooled and the coproporphyrin extracted quantitatively. The original publication should be consulted for details of the procedure (5, 7). Extraction was repeated eight to ten times with large amounts of acetic acid and of ether. A photoelectric colorimeter was used for the quantitative measurements. A 1.0 mg. per cent solution of crystalline coproporphyrin I in 5 per cent HCl was used as a standard. This was supplied through the kindness of Professor Hans Fischer. After measurement all the solutions containing coproporphyrin were combined, and the coproporphyrin was esterified and repeatedly recrystallized. Determinations of the melting point were then made in order to identify absolutely the type of the coproporphyrin present (7).

Determinations of the amount of hemoglobin in the eggs have been made essentially by the method described by Anson and Mirsky (26) for the measurement of heme in yeast. The embryonic areas were isolated from the eggs and combined. The hemoglobin was reduced and converted to pyridine-hemochromogen. To 1.0 cc. of egg yolk were added 3.0 cc. of distilled water, 1.0 cc. of 0.2 N NaOH, 1.0 cc. of pyridine, and a trace of sodium sulfite. The solution was then examined by a hand spectroscope, the intensity of the  $\alpha$  band being compared with the same band in solutions of embryo-free egg yolk containing an approximate known amount of hemoglobin. The amount of hemoglobin mixed with the egg yolk solution was measured from oxygen capacity

TABLE I  
*Coproporphyrin I of Chicken Embryo during Incubation*

Incubation period	No. of eggs	Total amount of coproporphyrin I	Coproporphyrin I Per embryo
<i>days</i>		<i>micrograms</i>	<i>micrograms</i>
3	21	Trace	Trace
9	28	41	1.4
10	15	31.5	2.1
11	22	41.5	1.9
13	17	65.0	3.8
15	10	32.5	3.3
16	18	90.0	5.0
17	13	55.0	4.2
18	8	42.0	5.3
18	9	46.9	5.1
19	17	101.0	5.9
20	14	94.5	6.8
21	9	67.5	7.5
			Per chick
25 1 day-old chickens*		165.0	6.6
25 1 " " " *		171.0	6.8

\* Fasted.

TABLE II  
*Hemoglobin of Chicken Embryo during the First 10 Days of Incubation*

Incubation period	Hemoglobin per embryo
<i>days</i>	<i>mg.</i>
1½	Trace
3	0.2
4	0.8
5	2.0
6	4.3 (3.9, 2.4, 4.8)
8	15.7 (16.9, 14.2, 16.1)
9	18.8 (18.1, 18.8, 19.5)
10	21.3 (20.6, 22.0)
	Total blood hemoglobin*
2 day-old chicken	180.0
2 " " "	165.0

\* These values were obtained by bleeding the animal to death, at the same time injecting saline to obtain as much blood as possible.

determinations, each cc. of oxygen capacity representing approximately 0.756 gm. of hemoglobin.

### *Results*

The results of the quantitative measurements of the content of coproporphyrin I of chick embryos in various stages of development are shown in Table I. Table II presents the content in hemoglobin of chick embryos for each day of incubation up to the 10th day, when, from the work of Sendju (25), it is known that bile pigment is present. On the 3rd day traces of coproporphyrin I and a measurable amount of hemoglobin, 0.2 mg., are present. From the 3rd to the 9th day the hemoglobin increases rapidly to 18.8 mg., and coincidentally the content of coproporphyrin I increases to 1.9 micrograms. Sendju's (25) value for hemoglobin in the 7 day-old chick embryo of 7.5 mg. falls nearly on the curve. The same author found 141 mg. of hemoglobin in 19 day-old embryos. Studies of the total hemoglobin content of the embryos were not made past the 10th day, since a possible conversion of Type III porphyrin could not be excluded because of the presence of bile pigment. A steady increase in the amount of coproporphyrin I present in each embryo was apparent, however, from 1.9 micrograms on the 11th day to 7.5 micrograms on the 21st day. By bleeding 2 day-old chickens to death a minimum value of 170 mg. for blood hemoglobin was obtained.

Study of the content of the egg-shell during incubation showed no detectable amounts of coproporphyrin I. The coproporphyrin methyl ester was identified as coproporphyrin I by melting point determinations. After three recrystallizations the melting point was 234–236° and after the fourth, 250°. Coproporphyrin III was not obtained in any of the fractions.

### DISCUSSION

The experimental results show that coproporphyrin I is present in the chick embryo and appears almost coincidentally with the Type III porphyrins of hemoglobin. An increased amount of Type III porphyrin occurs with increased amount of coproporphyrin I. Since egg-shells contain no detectable amounts of coproporphyrin I, any exogenous origin of the latter is impossible. It appears unlikely that coproporphyrin I can be derived by the

degradation of Type III porphyrins to simple pyrroles followed by a resynthesis of the latter into Type I compounds. No evidence of such a conversion is available.

The analytical values for the amount of hemoglobin found are possibly too low as a measure of the quantity of protoporphyrin produced, since a part of the protoporphyrin may still not have been utilized for hemoglobin formation. If the total amount of free protoporphyrin were estimated, it would be necessary to deduct from the total the amount of porphyrin preexisting in the shell (protoporphyrin), which van den Bergh and Grotepass (24) have shown diffuses in from the shell during incubation.

From the facts presented it is not possible to confirm the existence of a constant ratio between the production of Type III porphyrin (determined as hemoglobin) and Type I coproporphyrin, as suggested by Dobriner. It is perhaps possible, as indicated by Dobriner, that Type III and Type I porphyrins are produced simultaneously with coproporphyrin I as a by-product, but the data in this paper indicate that the two substances are produced by an independent synthesis, as suggested by Fischer.

#### SUMMARY

1. Coproporphyrin I appears in the developing chick embryo nearly coincidentally with the appearance of hemoglobin (Type III porphyrin).
2. The amount of coproporphyrin I increases with the increase of hemoglobin.
3. Coproporphyrin I is derived neither from an exogenous source nor from converted Type III porphyrin.
4. The quantitative relationship between the two types of porphyrins is discussed.

The author is indebted to Dr. Konrad Dobriner for carrying out the crystallization and melting point determinations of the coproporphyrin I.

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## THE INTERACTION OF VITAMIN B<sub>1</sub> IN ENZYMIC REACTIONS

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Recently I have reported the synthesis of cocarboxylase from vitamin B<sub>1</sub> and orthophosphate by dry bakers' yeast freed of natural cocarboxylase, and by a duodenal enzyme preparation (1, 2). Lohmann and Schuster (3) have isolated cocarboxylase in crystalline form from bottom yeast. They found that cocarboxylase is a pyrophosphoric ester of vitamin B<sub>1</sub> (4). Simultaneously and independently of my report (1), there appeared preliminary notes on the enzymic synthesis of cocarboxylase by von Euler and Vestin (5) and by Kinnersley and Peters (6). These investigators used untreated yeast as the source of enzyme material.

Confirming the work of Lohmann and Schuster, Stern and Hofer (7) synthesized cocarboxylase from vitamin B<sub>1</sub> and POCl<sub>3</sub>, showing by cataphoretic experiments that esterification of the vitamin took place and that cocarboxylase is a diphosphoric ester of vitamin B<sub>1</sub>.

Because of the important rôle that cocarboxylase plays in carbohydrate metabolism, *e.g.* in the decarboxylation and dehydrogenation of pyruvic acid, I have continued the studies on cocarboxylase. Conditions for synthesis by the duodenal enzyme preparation have been studied and an attempt has been made at isolation of the ester in the pure state. The enzymic hydrolysis of the coenzyme has also been investigated and correlated by feeding experiments with vitamin B<sub>1</sub>.

### EXPERIMENTAL

#### *Preparation of Materials*

*Dry Duodenal Mucosa*—Pig duodenum was thoroughly washed with large quantities of tap water and then with hot distilled



water. The mucosa was scraped off, minced, and extracted three times with 7 times its weight of acetone, each extraction being carried out by shaking the mixture for 10 minutes in an Erlenmeyer flask. The defatted mucosa was dried overnight at room temperature, and ground to a fine powder.

*Dry Yeast*—200 gm. of Fleischmann's yeast were spread out on a glass plate and dried at 38° in a current of air. The drying was complete in 4 hours. If a drying apparatus is not available, the yeast may be dried at room temperature. The drying will then require from 3 to 4 days. The dry yeast was placed in a flask and stored in a refrigerator. It keeps for many months without losing its carboxylase or cocarboxylase activity.

*Cocarboxylase-Free Dry Yeast*—The following method for preparing cocarboxylase-free yeast is a modification of the procedure of Lohmann and Schuster (3); their method does not yield a carboxylase preparation completely free of cocarboxylase.

2 gm. of dry yeast were placed in a 250 cc. centrifuge flask. 100 cc. of 0.1 M Na<sub>2</sub>HPO<sub>4</sub> (at room temperature) were added. The flask was stoppered and shaken by hand for 12 minutes. The mixture was centrifuged and the supernatant fluid discarded. The washing with the phosphate solution was repeated once more for 12 minutes. Then the yeast was washed once with 100 cc. of distilled water by shaking for 3 minutes. The washed yeast was then suspended in 20 cc. of Sørensen's phosphate of pH 6.2 and was used in the following experiments as the cocarboxylase-free carboxylase. It may be seen from the first experiment of Table III that it contains no cocarboxylase when tested with the pyruvate solution.

*Pyruvate Solution*—A solution of sodium pyruvate containing 10 mg. of pyruvic acid and 0.2 mg. of magnesium as MgCl<sub>2</sub> per cc. was prepared and adjusted to pH 6.2 with NaOH.

*Estimation of Cocarboxylase Activity*—The arrangements were similar to those of Lohmann and Schuster (3). The Warburg-Barcroft apparatus was employed. The temperature was 35°. 0.5 cc. of the pyruvate solution was placed in the side arm.

#### *Synthesis of Cocarboxylase by Duodenal Enzyme Preparation under Various Conditions*

*Experiment 1*—1 gm. of duodenal enzyme preparation was placed in a sterile 125 cc. Erlenmeyer flask and 19 cc. of distilled

water and 10 mg. of vitamin B<sub>1</sub> (Merck's synthetic) in 1 cc. of distilled water were added. The various flasks were stoppered with cotton and incubated at 33° for 20 hours. This and the samples to follow were then boiled for 4 minutes and filtered. The solutions employed in these experiments were sterilized by filtration through a Berkefeld (bacterial) filter and as far as it was possible sterile conditions were maintained.

*Experiment 2*—In this experiment 19 cc. of Sørensen's orthophosphate of pH 6.8 were employed. Other conditions were the same as in Experiment 1.

*Experiment 3*—This sample, containing 19 cc. of phosphate of pH 6.8, 1 gm. of duodenal enzyme preparation, and 10 mg. of vitamin B<sub>1</sub>, was adjusted to pH 9.5 with sodium hydroxide. After 20 hours at 33° the pH of the sample changed to 8.2 owing to autolysis.

*Experiment 4*—This experiment was similar to Experiment 3; the sample was adjusted, however, to pH 8.2 and remained so after 20 hours.

*Experiment 5*—In this control experiment 19 cc. of orthophosphate of pH 6.8 and 1 gm. of duodenal enzyme preparation were used. The vitamin was added only after 20 hours at 33° and then the mixture was boiled for 4 minutes, filtered, and tested together with the other samples.

The results of these experiments are incorporated in Table I. It may be seen that there is considerable cocarboxylase synthesis on both sides of the pH scale, as well as in the absence of phosphate. It appears that the autolyzing tissue furnishes enough phosphate ions for esterification. The optimum of the reaction is at pH 6.8.

The active cocarboxylase solutions were adjusted to pH 6.2, filtered through a porcelain bacterial filter, and stored in sterile containers. Bacteria destroy cocarboxylase. Cell poisons, such as toluene, chloroform, etc., completely inhibit the action of the enzyme phosphatase. Glycerol has a similar action. Bacteria-free cocarboxylase is very stable. It may be preserved with toluene. Traces of toluene have no effect on the enzyme carboxylase.

Similar experiments were carried out with 1 mg. of MgSO<sub>4</sub>, 10 mg. of calcium hexosediphosphate, and sodium pyrophosphate. None of these substances, however, had any effect on the synthesis of cocarboxylase.

*Purification of Cocarboxylase*

To 100 cc. of cocarboxylase solution obtained by synthesis from the duodenal enzyme preparation (Table I, Experiment 1), 50 cc. of a 4 per cent neutral lead acetate solution were added. The mixture was centrifuged and the supernatant fluid discarded. The precipitate was suspended in 40 cc. of distilled water, H<sub>2</sub>S was passed through, and the lead sulfide was centrifuged off. The H<sub>2</sub>S and all the fluid were removed in a vacuum at room temperature. The dry residue was dissolved in 0.1 N HCl adjusted to pH 6.2 with N NaOH and the insoluble impurities were centrifuged off.

TABLE I  
*Showing Phosphorylation of Vitamin B<sub>1</sub> at Various pH Values*

Experiment No.	CO <sub>2</sub> in 20 min.	CO <sub>2</sub> in 40 min.	pH
	<i>c.mm.</i>	<i>c.mm.</i>	
1	110	166	6.55 (H <sub>2</sub> O)
2	130	200	6.8
3	60	105	9.5-8.2
4	88	150	8.15
5	0	-1	6.8

Orthophosphate was used in Experiments 2 to 5. In all manometric tests described in this paper 1 cc. of washed dry yeast in phosphate of pH 6.2 and the solutions to be tested for cocarboxylase activity were placed in the main compartment of the Warburg vessels. In each experiment 0.5 cc. of cocarboxylase was used. 0.5 cc. of sodium pyruvate (5 mg. of pyruvic acid containing 0.1 mg. of magnesium as MgCl<sub>2</sub>) was employed as a substrate and placed in the side arm of the vessels. H<sub>2</sub>O was added to make a total of 3 cc. The temperature was 35°.

While all pigments and most impurities are removed by this procedure, the product is not pure yet.

*Properties of Purified Cocarboxylase*—The purified cocarboxylase may be oxidized to thiochrome by sodium hydroxide and ferri-cyanide. It is hydrolyzed and inactivated when boiled with N HCl for 10 minutes. It is also hydrolyzed by kidney tissue but not by the duodenal enzyme preparation. Addition of the duodenal enzyme preparation to the purified cocarboxylase and incubation for 24 hours at 38° at various hydrogen ion concentra-

tions do not inactivate the coenzyme. On the contrary, if the samples contained unphosphorylated vitamin B<sub>1</sub>, an increase in cocarboxylase activity took place. Nor did hydrolysis occur when MgSO<sub>4</sub> (10 mg. per 20 cc. and per gm. of mucosa preparation) was added to the samples at various hydrogen ion concentrations.

#### *Hydrolysis of Cocarboxylase by Kidney Tissue*

Since the duodenal enzyme preparation does not hydrolyze cocarboxylase, even in the absence of phosphate which is known to inhibit phosphatolysis, and because the ingestion of large amounts of vitamin B<sub>1</sub> does not result in cocarboxylase excretion (see below), kidney tissue was employed.

*Sample A*—5 cc. of cocarboxylase (synthesized in H<sub>2</sub>O), 10 mg. of MgSO<sub>4</sub> (in 1 cc. of H<sub>2</sub>O), 5 cc. of distilled water, and 4 gm. of beef kidney tissue minced three times were placed in a 50 cc. Erlenmeyer flask.

*Sample B*—This flask contained 5 cc. of the cocarboxylase and 10 mg. of MgSO<sub>4</sub> only.

*Sample C*—In this flask were placed 4 gm. of the minced kidney tissue in 5 cc. of distilled water.

*Sample D*—This sample contained 4 gm. of kidney tissue in 10 cc. of water and 10 mg. of MgSO<sub>4</sub>.

The four samples were kept at 38° for 3½ hours. At the end of incubation Samples B and C were mixed, boiled for 4 minutes, and centrifuged. This mixture served as a control and was compared with Samples A and D after they were boiled for 4 minutes and centrifuged.

It may be seen from Experiment 1 of Table II that kidney tissue hydrolyzed more than 50 per cent of the cocarboxylase in 3½ hours at 38°. The kidney tissue itself showed no cocarboxylase activity under the conditions of the experiment (Sample D).

It should be noted that there is no direct proportionality between cocarboxylase concentration (activity) and CO<sub>2</sub> formation (see Table III). The decarboxylation of pyruvic acid is not governed by the cocarboxylase. If the cocarboxylase concentration is increased 3 times, CO<sub>2</sub> formation is only doubled after the first 15 minutes of the experiment. This corroborates the findings of others (3).

*Ingestion of Large Quantities of Vitamin B<sub>1</sub> and Its Excretion*

In order to find out whether the ingestion of large quantities of vitamin B<sub>1</sub> results in the excretion of the pyrophosphate, 50 mg. (10,000 international units) of vitamin B<sub>1</sub> in 250 cc. of water were ingested by three healthy men about 50 kilos in body weight. No ill effects were observed. The taste and odor of the vitamin,

TABLE II

*Showing Hydrolysis of Cocarboxylase by Kidney Tissue*

Temperature 38°. After 3½ hours all samples were boiled for 4 minutes, filtered, and tested for cocarboxylase activity.

Experiment No.		CO <sub>2</sub> in 20 min.	CO <sub>2</sub> in 40 min.
		c.mm.	c.mm.
1	0.5 cc. filtrate, boiled Sample A containing 5 cc. cocarboxylase, 5 cc. H <sub>2</sub> O, and 4 gm. kidney tissue	48	78
2	0.5 cc. filtrate, mixture of Sample B containing 5 cc. cocarboxylase, and Sample C containing 5 cc. H <sub>2</sub> O and 4 gm. kidney tissue; both samples kept separate at 38° and mixed after 3½ hrs.	83	130
3	0.5 cc. filtrate, boiled Sample D containing 4 gm. kidney tissue in 10 cc. H <sub>2</sub> O	-2	-3

TABLE III

*Showing Effect of Increasing Amounts of Cocarboxylase*

Cocarboxylase added	CO <sub>2</sub> in 15 min.	CO <sub>2</sub> in 25 min.	CO <sub>2</sub> in 35 min.
cc.	c.mm.	c.mm.	c.mm.
None	-1	-2	-2
0.3	27	44	51
0.6	40	60	78
0.9	53	76	89

however, were noticed for many hours. The vitamin appeared suddenly in the urine, most of it being excreted at one time. The following details will illustrate the observation. In one of the three subjects studied the vitamin appeared 2 hours after ingestion and was mostly excreted within 7 hours. In the second case the vitamin appeared in the urine 5 hours after ingestion, with maxi-

mun excretion between 8 and 17 hours. In the third case, however, the vitamin was excreted only after 24 hours, when it appeared in high concentration for the next 4 hours.<sup>1</sup>

The various samples of urine were adjusted to pH 6.2 and tested for cocarboxylase activity. They were all inactive. The test for cocarboxylase is a very sensitive one, as fractions of a microgram may readily be detected. Apparently the kidneys dephosphorylate the vitamin before it is excreted.

While no toxic effects were noticed following the ingestion of a single large dose of vitamin B<sub>1</sub>, Perla (8) has recently shown that continuous use of excessive amounts of vitamin B<sub>1</sub> has toxic effects on rats.

#### DISCUSSION AND SUMMARY

The experiments show that the duodenal enzyme preparation has the ability to convert vitamin B<sub>1</sub> into cocarboxylase (vitamin B<sub>1</sub>-pyrophosphate) under a variety of conditions. This enzyme preparation, however, cannot hydrolyze the coenzyme. It appears that the phosphorylation is catalyzed by an enzyme or enzyme system, phosphatase, different from the phosphatases which cause hydrolysis of esters of phosphoric acid. Cocarboxylase, however, is rapidly hydrolyzed by kidney tissue and by boiling dilute HCl.

Partial purification of the coenzyme had been obtained by precipitating the vitamin B<sub>1</sub>-pyrophosphate in the form of its lead salt and liberation of the ester by H<sub>2</sub>S.

Ingestion of large amounts of vitamin B<sub>1</sub> did not result in the excretion of the ester. Most of the vitamin appeared in the urine within a certain definite period. Obviously the kidneys dephosphorylate the vitamin before it is removed.

Improvements for the preparation of enzyme materials employed have been described.

While in plants much of the vitamin B<sub>1</sub> is present in the unphosphorylated state (9), mammalian tissues contain all of the vitamin as the pyrophosphoric ester (10). Lipmann (10) has shown that in mammalian tissues cocarboxylase functions as the coenzyme for a specific pyruvic acid dehydrogenase. Thus the conversion

<sup>1</sup> I am grateful to Dr. R. R. Williams and Dr. J. K. Cline for identifying the vitamin B<sub>1</sub> in the urine samples.

of vitamin B<sub>1</sub> to the active coenzyme (vitamin B<sub>1</sub>-pyrophosphate) appears to be of great physiological importance.

I am indebted to Merck and Company, Inc., through the kindness of Dr. R. T. Major, for generous supplies of their synthetic vitamin B<sub>1</sub>.

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## STREAM DOUBLE REFRACTION OF VIRUS PROTEINS

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Double refraction of flow was observed as early as 1874 by Maxwell (see Ambronn and Frey (1)) who showed that Canada balsam becomes optically anisotropic when it is stirred vigorously. Some years later Kundt found that gelatin sols show this phenomenon (1). Freundlich (7) observed that sols of vanadium pentoxide and of certain dyes show stream double refraction. He further pointed out that the particles of the vanadium pentoxide sols are rod-shaped, and concluded that the double refraction was due to the orientation of the rod-shaped particles in the viscous stream. More recently, von Muralt and Edsall (13) have shown that myosin, the globulin of muscle, shows the phenomenon to a marked extent. Ovoglobulin and egg albumin gel, the latter at high pH values, have been reported by Boehm and Signer (5) to show stream double refraction. Takahashi and Rawlins (19) have reported that the juice expressed from tobacco plants infected with tobacco mosaic virus exhibited this property, and more recently they have shown that purified tobacco mosaic virus protein behaves similarly. Finally, other plant virus proteins, those of aucuba mosaic of tomato, enation mosaic of tomato, and Ainsworth's cucumber mosaics Nos. 3 and 4, have been shown by Bawden and Pirie (2) to exhibit double refraction of flow. These workers regard all of these viruses as being related to that of tobacco mosaic.

Because of Freundlich's findings, Takahashi and Rawlins regarded the double refraction of flow in tobacco mosaic virus solutions as being due to the orientation of asymmetrical particles, and, by a method which will be mentioned later, limited this to the orientation of rod-shaped particles. This interpretation of the



results has been followed in the publications of other workers. However, as will be seen in the next section, double refraction of flow may be the result of other causes than orientation of rods. Therefore, it was thought desirable to consider this question further and to extend the investigation to include other virus proteins and proteins known to be asymmetrical in shape.

### *Theory of Stream Double Refraction*

A doubly refracting or optically anisotropic body is one which is capable of transmitting light in such a manner that all of the electrical disturbances are distributed within two planes at right angles to each other. A beam of light in which all of the electrical disturbances are in one plane is said to be plane-polarized. A doubly refracting crystal, then, is one which transmits light, part or all of which is polarized in one plane and the remainder of which is polarized in a plane at right angles to the first. A body which simultaneously transmits light polarized in two planes at right angles to each other is said to transmit elliptically polarized light. With the exception of the very special case where one of the transmission directions of the doubly refracting crystal coincides with the plane of polarization of the incident light, even originally plane-polarized light is changed to elliptically polarized light by anisotropic crystals. Elliptically polarized white light can never be completely extinguished by a nicol prism. Therefore, when a doubly refracting crystal is placed between two crossed nicols, some light will be transmitted by the second nicol for every position of the doubly refracting crystal except those in which one of the transmission planes is parallel to the plane of polarization of light from the first nicol. This effect will be at a maximum when the crystal is rotated about the beam through an angle of  $45^\circ$  from the position just mentioned. If a  $V_2O_5$  sol is caused to flow through a tube placed between two crossed nicols in such a manner that the direction of flow is perpendicular to the path of the light traveling from polarizer to analyzer and makes an angle of  $45^\circ$  with the transmission plane of either nicol, that portion of the field intercepted by the capillary tube will appear to be illuminated, whereas the remainder of it will be dark. When flow ceases, the whole field becomes dark.

It is generally agreed that double refraction of flow in a solution may be due to one or more of three fundamental causes. The first possible cause lies in what is described as the "photoelastic effect," a discussion of which is given by Kunitz (11). When amorphous materials such as glass, celluloid, etc., are subjected to stresses, they become doubly refracting at points of strain. If a material must be deformed in order to make it flow, internal strains will develop and these can be the cause of double refraction. In this type of anisotropy, the effect should cease immediately after the stress is relieved. Highly viscous gel-like materials which exhibit double refraction of flow may be said to do so because of this photoelastic effect.

The second possible cause lies in the orientation of rod-like or plate-like isotropic particles in the viscous stream. A mathematical discussion of this question is given by Kuhn (10). The case of a suspension or solution of small needle-shaped bodies in a viscous medium flowing through a small capillary tube of circular cross-section may be considered. Those molecules of liquid which are adjacent to the walls of the tube will be at rest, while those in the center of the tube will be moving at the greatest velocity. There will be a velocity gradient in all radial directions. A needle-shaped particle oriented radially will have one end in a layer of greater velocity than the other. This will exert a torque on the particle, tending to orient it in a direction parallel to that of the flow. Tending to randomize the orientation will be the effect of Brownian movement. Increased length and increased weight of the needle-shaped particle will act to render this randomizing tendency less effective; *i.e.*, the scatter of the actual orientation of individual particles about the mean orientation in the direction of flow will be reduced. Long, heavy rods should orient well in a flowing viscous medium, and in this case there should be an appreciable time interval between the cessation of streaming and the complete randomization of the rods.

Wiener (20) (see also Ambronn and Frey (1)) has shown from a theoretical point of view that isotropic rods, small compared to the wave-length of light, oriented parallel to each other, and suspended in an isotropic medium of different refractive index,

constitute an optically anisotropic system. The following equation describes the double refraction of such a system

$$N_e^2 - N_o^2 = \frac{V_1 V_2 (N_1^2 - N_2^2)^2}{(V_1 + 1) N_2^2 + V_2 N_1^2}$$

where  $N_e$  and  $N_o$  are the refractive indices for the extraordinary and the ordinary beams, and  $N_1$  and  $N_2$  the refractive indices of the rods and the medium, and  $V_1$  and  $V_2$  the relative volumes of the rods and of the medium, respectively. In the case of a dilute suspension of rods ( $V_2 = 1000 V_1$ ), with  $V_1 + V_2 = 1$ , this equation reduces to

$$(N_e - N_o)(N_e + N_o) = \frac{V_1 V_2 (N_1^2 - N_2^2)^2}{V_2 (N_1^2 + N_2^2)}$$

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\* This equation is obtained from Equation 314, p. 581, of the paper by Wiener (20).

$$E_m = \frac{E_1 E_2 + M(V_1 E_1 + V_2 E_2)}{M + V_2 E_1 + V_1 E_2}$$

where  $V_1$  and  $V_2$  refer to the relative volumes of the first and second components,  $E_1$ ,  $E_2$ , and  $E_m$  refer to the dielectric constants of the first and second components, and of the whole system, respectively, and  $M$  is a factor whose magnitude depends upon the shape of the suspended particles. If we are dealing with long thin isotropic rods oriented parallel in an isotropic medium,  $M$  approaches  $\infty$  for electric fields in a plane parallel to the direction of orientation, and  $M$  approaches  $E_2$  for fields in a plane perpendicular to that one. For parallel fields,  $E_m = V_1 E_1 + V_2 E_2 = E_o$ . For perpendicular fields

$$E_m = \frac{(1 + V_1)E_1 + (1 - V_1)E_2}{(1 - V_1)E_1 + (1 + V_1)E_2} \times E_2 = E_o$$

$E_e - E_o$

$$= \frac{(V_1 E_1 + V_2 E_2)((1 - V_1)E_1 + (1 + V_1)E_2) - ((1 + V_1)E_1 + (1 - V_1)E_2)E_2}{(1 - V_1)E_1 + (1 + V_1)E_2}$$

With use of the relationship  $V_1 + V_2 = 1$ ,

$$E_e - E_o = (V_1 V_2 (E_1 - E_2)^2) / ((V_1 + 1)E_2 + V_2 E_1)$$

From the electromagnetic theory of light,  $N^2 = E$ . Substituting in the above equation, we obtain

$$N_e^2 - N_o^2 = (V_1 V_2 (N_1^2 - N_2^2)^2) / ((V_1 + 1)N_2^2 + V_2 N_1^2)$$

Since  $N_e$  and  $N_o$  are both large compared to the double refraction ( $N_e - N_o$ ), ( $N_e + N_o$ ) will not change appreciably even when ( $N_e - N_o$ ) varies over a wide range. Therefore, we may write as a close approximation

$$(N_e - N_o) = \frac{KV_1(N_1^2 - N_2^2)^2}{N_1^2 + N_2^2}$$

where  $K$  is a constant. Hence, the double refraction of a system involving the orientation of isotropic particles is, in the limiting case, directly proportional to the relative volume and, therefore, the concentration of the rods suspended within the optical path. Double refraction of this sort must always be positive with the optic axis in the direction of streaming.

The third possible cause of double refraction of flow lies in the orientation of doubly refracting rods or plates. Obviously, if anisotropic crystals are all oriented the same way in an isotropic medium, the whole optical effect will be additive and the system as a whole will exhibit double refraction. In such a case too the double refraction of the suspension would be proportional to the concentration of the suspended particles. Furthermore, double refraction of the type discussed by Wiener would also obtain in such systems, so that the total double refraction would be the sum of two terms. When submicroscopic anisotropic particles revert to a state of random orientation, a statistical isotropy obtains, because the number of particles in the path of a very fine beam of light is sufficiently large to insure practically complete compensation.

In conjunction with this, it should be pointed out that, if the doubly refracting, randomly oriented crystals are of microscopic size or larger, one would not expect to obtain statistical isotropy in a system of small dimensions. The case of a suspension of anisotropic crystals of microscopic size in an isotropic medium contained in a vessel of volume  $lS$ , where  $l$  is the depth of the vessel and  $S$  is the area of a face perpendicular to the direction of  $l$ , may be considered. This volume may be regarded as being composed of a great number of small volumes, each being  $l dS$ . If light passes through the vessel in the direction of  $l$ , we will find that for every element of volume  $l dS$  there will be a small residual double refraction. This will be realized because the number of

randomly oriented, doubly refracting crystals in the path of a beam of light of cross-section  $dS$  will not be great enough to insure absolute compensation at any instant of time. At any given time, each element of volume will behave like a crystal with its own value of double refraction and its own extinction directions. Placed between crossed Polaroid plates in such a manner that light will pass through the system in the direction of  $l$ , the whole system  $lS$  will appear to be doubly refracting, because scattered all through it will be doubly refracting elements,  $l dS$ , oriented so as to transmit light. In another instant some of these will no longer have the proper orientation, but others will have taken their place. If one rotates the polarizer and analyzer together about the beam, one will find no extinction directions, because extinction directions of the elements  $l dS$  are randomly distributed at any instant. For every element extinguished by rotating the analyzer and polarizer a new one will begin to transmit. We are dealing, then, with a system which is doubly refracting because at every instant of time it appears to transmit light when it is viewed with crossed Polaroid plates, but whose double refraction differs from that of a perfect calcite crystal because there are no extinction directions.

Freundlich (7) was able to show with the ultramicroscope that vanadium pentoxide particles are needle-shaped. Furthermore, Farwell (6) has recently shown that double refraction of flow in  $V_2O_5$  sols persists for a short time even after the sol has passed from the end of the tube into the air. This means that the double refraction is not dependent upon the internal strains within the flowing material, and therefore eliminates the photoelastic effect as the sole cause of double refraction of flow in  $V_2O_5$  sols. Von Muralt and Edsall (13) have observed a time lag between cessation of streaming and assumption of the isotropic state in the case of muscle globulin. This, of course, would correspond to the time needed for complete randomization of rod-shaped particles. Takahashi and Rawlins (19) interpreted their observations to mean that the infective agent of the tobacco mosaic disease was composed of or associated with rod-shaped particles. They eliminated the possibility of their being plate-like particles by showing that the entire path of the flowing infective juice was anisotropic, whereas only the edges of the stream were anisotropic

when a suspension of plate-like particles (ferric oxide sol) was caused to flow. In the discussion of the data presented in this paper, further evidence for the proposed rod-like shape of tobacco mosaic virus protein particles will be considered.

### *Method*

A good discussion of various methods of measuring stream double refraction is presented by von Muralt and Edsall (13). The method to be discussed in this paper is a modification of the one used by Takahashi and Rawlins (19). These workers simply caused the liquid under investigation to flow through a capillary tube into a body of the same liquid on the stage of a polarizing microscope. The apparatus was so arranged that the end of the capillary tube was in the optical field and that the direction of flow was at an angle of  $45^\circ$  to one of the two crossed nicol prisms. Their method was used for quantitative studies by merely diluting the material under investigation until double refraction of flow could no longer be detected by means of direct observation. The method here presented is in principle the same, but modified in detail so as to become almost entirely mechanical. Fig. 1 shows a picture diagram of the apparatus used. White light from an incandescent tungsten filament bulb is reflected into a polarizing nicol prism so fixed that its plane of polarization makes an angle of  $45^\circ$  with the plane of the paper. The emerging plane-polarized light passes through the substage condenser into the streaming cell, which consists of a piece of flat glass tubing whose internal dimensions are about  $1 \times 7 \times 25$  mm. The tube is so arranged that the light must pass through 7 mm. of streaming liquid and that the direction of streaming is parallel to the plane of the paper. A small quantity of liquid (10 cc. or less) is forced back and forth through the cell by a small air pump made from a hypodermic syringe and operated by a small electric motor whose angular velocity may be controlled with a rheostat. The light then passes through a low power objective into the microscope tube where it meets another nicol prism, the analyzer, whose plane of polarization is at right angles to that of the polarizer. That portion of the elliptically polarized light which is transmitted by the analyzer passes through an eyepiece lens and then strikes the cathode of a sensitive vacuum type photoelectric tube. As the

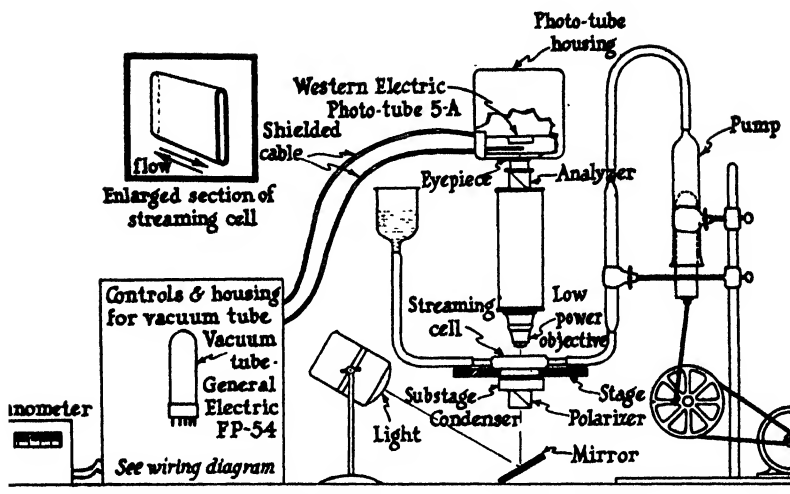
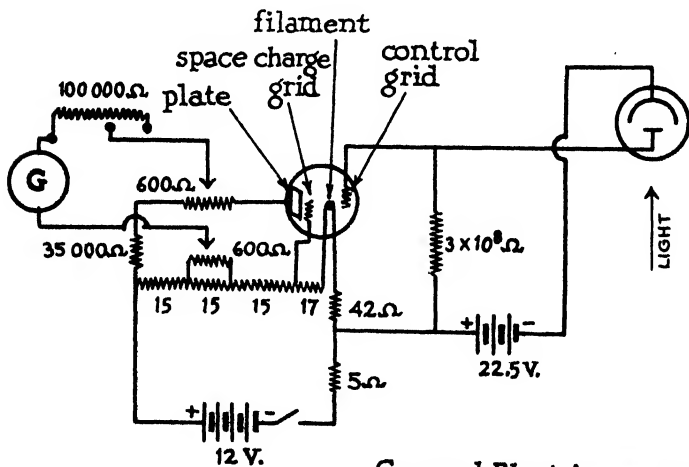


FIG. 1. Diagrammatic picture of apparatus



General Electric vacuum  
tube FP-54

Western Electric photo-tube 5-

FIG. 2. Wiring diagram of electrical circuit of apparatus

wiring diagram shown in Fig. 2 indicates, the current thus set up is allowed to leak through a resistor of  $3 \times 10^8$  ohms. The almost negligible current of  $10^{-9}$  ampere would set up a potential difference of 0.3 volt across the resistor and would thereby alter the potential on the control grid of the vacuum tube by that amount. A very small fraction of this change is sufficient to cause a change in the plate current in a tube of the type employed. As the diagram indicates, changes in the plate current are detected by use of a box type galvanometer, sensitive to 0.025 microampere, in a bridge circuit. Scale deflections of the galvanometer during streaming are taken as a measure of the change in light flux caused by changing the system from an isotropic one to an anisotropic one.

An inspection of Fig. 3 will show that the galvanometer deflections are, in the lower concentration ranges, approximately proportional to the concentration of any given virus protein preparation. As may be seen from the theory of Wiener, the double refraction should be proportional to the concentration of rod-shaped particles in the low concentration regions. This leads one to believe that, as concentrations of virus protein approach zero, the galvanometer deflections are proportional to the actual double refraction of flow of the protein solutions.

This linear relationship of galvanometer reading to protein concentration is a very fortunate one, though entirely without theoretical significance. It may be seen in Johannsen's book (9) that for white light,

$$I = \sum r_i^2 \sin^2 2\theta \sin^2 \left( \frac{\pi(N_2 - N_1)M}{\lambda_i} \right)$$

where  $I$  is the intensity of light transmitted by the analyzer,  $r_i$  is the amplitude of a very small band of the incident beam of light of wave-length  $\lambda_i$ ,  $\theta$  is the angle between the plane of polarization of the polarizer and one of the transmission directions of the doubly refracting material,  $(N_2 - N_1)$  is the double refraction, and  $M$  is the thickness of the anisotropic body. The symbol  $\Sigma$  indicates that the intensities calculated for every portion of the spectrum by the above formula must be added together to get the total intensity,  $I$ . It is obvious that  $I$  bears no readily predictable linear relationship to the double refraction, and therefore the approximate linear dependence of galvanometer deflection on



concentration of virus and, hence, on double refraction must be regarded as being fortuitous.

The degree to which particles will be oriented will depend upon the rate of streaming. With a streaming system such as the one here used, the maximum rate of shear is obtained when the pump moves with just slightly greater than its minimum velocity. The

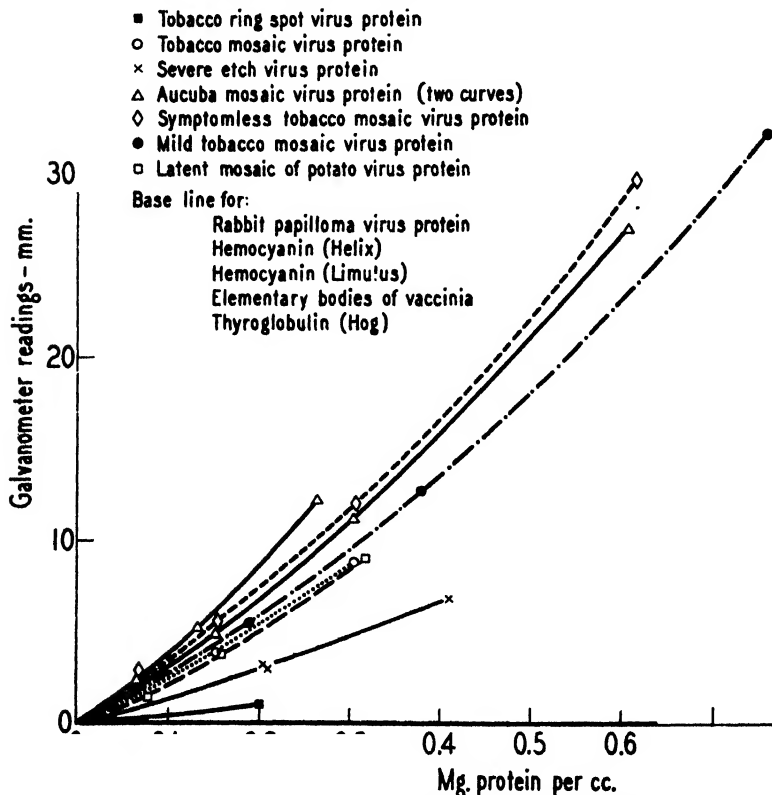


FIG. 3. Double refraction of flow of protein solutions

reason for this is obvious, for, if the pump is operated at too great a rate, there will be very little actual movement of liquid. The speed of maximum shear is easily recognized as the speed of maximum galvanometer deflection. This maximum is sufficiently broad that only very small errors result from the differences in pump velocities from reading to reading.

*Presentation and Discussion of Results*

Takahashi and Rawlins (19) first reported that the juice expressed from tobacco plants infected with tobacco mosaic virus showed double refraction of flow. That observation was repeated in this laboratory on juice expressed from plants previously frozen, ground, thawed, and then filtered through a celite filter. A similar observation was made on the juice of tobacco plants infected with the latent mosaic of potato. The double refraction of flow in this latter case was small compared to that of the juice of tobacco plants infected with tobacco mosaic virus, due probably to the fact that the concentration of latent mosaic virus protein in infectious juice is quite low. No sample of juice from healthy plants was found to show double refraction of flow. This observation confirms the results reported by Bawden and Pirie (2), but is not in accord with the finding of Takahashi and Rawlins that juice from healthy plants does sometimes show the phenomenon. It must be remembered, however, that negative results mean only that the double refraction of flow was either absent or not great enough to be detected in the samples examined.

It may be seen from Fig. 3 that all of the seven purified plant virus proteins studied show double refraction of flow. The solvent in all of these studies was 0.1 M phosphate buffer at pH 7. With the aid of an  $R_1$  gypsum plate, the double refraction of a flowing solution of tobacco mosaic virus protein was found to be positive with the optic axis in the direction of flow. Those proteins on the list not previously reported which show double refraction of flow are a protein isolated from Turkish tobacco plants infected with severe etch virus (16, 17), practically inactive at the time of measurement, the protein of the symptomless tobacco mosaic virus (Holmes) (8, 21), that of a mild strain of tobacco mosaic virus, that of tobacco ring spot virus (17), and that of the latent mosaic of potato virus<sup>1</sup> (12, 17). Two preparations of viruses affecting animals, the heavy protein material isolated from tissues diseased with the Shope papilloma virus<sup>1</sup> (4), and a purified

<sup>1</sup> It is a pleasure to acknowledge indebtedness to Dr. H. S. Loring for the latent mosaic virus protein, to Dr. J. W. Beard for the Shope papilloma virus protein, to Dr. C. V. Seastone for the vaccinia elementary bodies and *Limulus* hemocyanin, to Dr. A. Claude for the *Limulus* and *Helix* hemocyanins, and to Dr. M. Heidelberger for the thyroglobulin used in these experiments.

suspension of elementary bodies of vaccinia<sup>1</sup> (14), did not show sufficient stream double refraction to be detected under the conditions of the experiment. The double refraction of flow of *Limulus* and *Helix* hemocyanins<sup>1</sup> and of hog thyroglobulin<sup>1</sup> is represented by the base-line in Fig. 3. After increasing the sensitivity of the apparatus so that 0.03 mg. of tobacco mosaic virus protein per cc. gave a reading of 3.5 mm. on the galvanometer scale, it was found that all three of the above materials showed double refraction of flow. At this sensitivity, a *Limulus* hemocyanin solution containing 52.3 mg. of protein per cc. gave a reading of 1.5 to 2 mm.; *Helix* hemocyanin at a concentration of 7.97 mg. per cc. gave a reading of 3 mm.; and hog thyroglobulin at a concentration

TABLE I

*Variations in Stream Double Refraction of Tobacco Mosaic Virus Proteins*  
0.73 mg. of protein per cc. in 0.1 M phosphate buffer at pH 7.

Sample No.	Stream double refraction	Virus protein samples
1	25.7	2 yrs. old, chemically prepared, brown-colored
2	20.7	Recently chemically prepared, colorless
3	14.8	" ultracentrifuged, colorless
4	26.2	" 3 times ultracentrifuged, brown-colored
5	23.0	" 4 " " "
6	14.3	In supernatant of last centrifugation of Sample 5, colorless

of 5.3 mg. per cc. gave a reading of about 1 mm. On a protein basis, these materials show the effect to the extent of about 1/3000, 1/300, and 1/600, respectively, that of the tobacco mosaic virus protein. Dissymmetry constants of 1.3 and 1.5 have been assigned to *Helix* hemocyanin and thyroglobulin, respectively. (18). These results are given to emphasize the great extent to which the plant virus proteins show this property.

As may be seen from the results presented in Table I, the stream double refraction per unit concentration of tobacco mosaic virus protein varies considerably from preparation to preparation, apparently depending upon modifications in preparative technique. In Fig. 3 are given two curves for aucuba mosaic virus protein. Therefore, one is hardly justified in attaching significance to the

relative slopes of the curves in Fig. 3. It is significant that all of the plant virus proteins examined do show double refraction of flow to a remarkable degree.

Bawden and Pirie (2) have observed that inactivation of tobacco mosaic virus protein with x-rays or nitrous acid does not destroy the property of stream double refraction. In line with this observation, we have found that inactivation with formaldehyde and with hydrogen peroxide does not destroy this property of the virus proteins. Previously Wyckoff, Biscoe, and Stanley (21) and Stanley (15) had found that neither the sedimentation constant nor the ability of the tobacco mosaic virus protein to crystallize in needle-shaped crystals was changed by these treatments. This type of inactivation, then, does not seem to be associated with any profound change in the size and shape of the virus protein particles.

As was pointed out earlier, double refraction of flow in virus protein solutions could possibly be attributed to (a) photoelastic effect, (b) orientation of isotropic or anisotropic rods, or (c) orientation of isotropic or anisotropic plates.<sup>2</sup> The last possibility is easily ruled out by the approach used by Takahashi and Rawlins (19). We have observed, using the polarizing microscope, that the entire streams of all of the plant virus proteins considered in this study are doubly refracting. As was previously shown, if the stream double refraction were due to the orientation of isotropic or anisotropic plates, only the edges of the streams would show double refraction.

Evidence has now been obtained that the photoelastic effect is not the sole cause of the double refraction in plant virus protein solutions, for, if it were, the viscosity should be an important factor. Actually, we have found that double refraction of flow

<sup>2</sup> As was pointed out by Bear, Schmitt, and Young (3), the actual shape of the suspended particles could vary considerably from the ideal shapes considered by Wiener, and both rods and plates could approach ribbons in shape. Although the particles considered in this paper have been referred to consistently as rods, this designation should be interpreted as referring to any particles having one dimension considerably greater than the other two. Possibilities would include particles having elliptical, angular, or essentially flat cross-sections. The exact theoretical considerations for such possibilities have not been worked out, although Wiener's theory should hold in a qualitative way.

can be detected in solutions so dilute that their viscosities do not differ appreciably from that of pure water. Although decreasing the pH of dilute tobacco mosaic virus protein solutions from 7 to 5 causes a very marked increase in double refraction of flow, the relative viscosity increases but a few hundredths of its former value. Furthermore, when one works near the isoelectric point of the protein, the double refraction tends to decrease but slowly after streaming stops. This means that the anisotropy persists after the shearing strains have disappeared. This time lag could correspond to the time of randomization of oriented rods. It is in some ways analogous to the "relaxation times" encountered in studies of the dielectric properties of protein solutions (22). However, the most striking evidence against the possibility of the stream double refraction being due solely or even largely to the photoelastic effect is the fact that, when a solution of tobacco mosaic virus protein is allowed to flow from a pipette into the air, the double refraction persists for a time after the liquid leaves the end of the pipette. This is clearly shown in Fig. 4. A solution of tobacco mosaic virus protein in water containing 25 mg. of protein per cc. was allowed to flow into the air from the end of a 1 cc. pipette. It was photographed between two Polaroid plates, the planes of polarization of which made angles of  $45^\circ$  with the direction of flow and  $90^\circ$  with each other. Fig. 5 is a photograph of the same system between "parallel" Polaroid plates. Here the anisotropic stream appears to be dark, because part of the elliptically polarized light is screened out by the analyzer. It should be noted that this experiment differs distinctly from those performed by Takahashi and Rawlins (19) and those performed by Bawden and Pirie (2), all of whom caused streams of virus solutions to flow into larger bodies of the same solutions. In such a case the shear is about the same after the stream has passed beyond the end of the pipette. However, when the liquid flows from a pipette into the air, the shear beyond the end of the pipette is very greatly reduced, although not entirely eliminated (6). The fact that the double refraction disappears but slowly when the shear drops off abruptly eliminates the photoelastic effect as the sole cause of the double refraction. The rod theory serves well to explain the results, for the rods, oriented in the viscous streams, require a certain time in which to revert to the random state after the orienting force is greatly reduced.

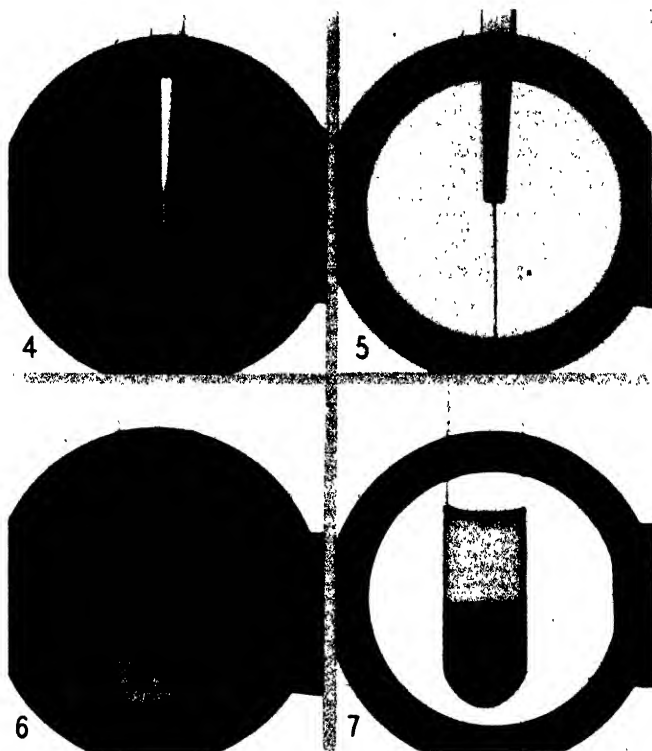


FIG. 4. Doubly refracting stream of tobacco mosaic virus protein solution flowing from the end of a pipette, photographed between crossed Polaroid plates arranged so that each vibration direction of the Polaroid plates makes an angle of  $45^\circ$  with the direction of flow. Photograph by J. A. Carlile.

FIG. 5. Same system as in Fig. 4 photographed between parallel Polaroid plates. The doubly refracting stream appears to be darker than the background in this case. Photograph by J. A. Carlile.

FIG. 6. A tube of tobacco mosaic virus protein solution which, upon standing, has separated into two layers. The photograph, taken with the aid of crossed Polaroid plates, shows that the bottom layer material is spontaneously doubly refracting, whereas the top layer material is not spontaneously doubly refracting. Double refraction of flow is shown by the proteins in both layers. Photograph by J. A. Carlile.

FIG. 7. The same system as in Fig. 6 taken between parallel Polaroid plates. The intensity of the light transmitted by the bottom layer is about the same as in the case represented in Fig. 6. Photograph by J. A. Carlile.

Bawden and Pirie (2) pointed out that, upon standing, relatively concentrated tobacco mosaic virus protein solutions separate into two layers, the bottom one of which is spontaneously birefringent. This behavior, they believe, means that the virus particles are rods, and that, when their concentration in the bottom layer becomes so great that they lose their ability to rotate about their diameters, they become spontaneously oriented parallel to each other. The observation of this phenomenon has been confirmed in this laboratory. Fig. 6 is a picture of a tube containing ultracentrifugally isolated tobacco mosaic virus protein in a concentration of about 60 mg. per cc., photographed between crossed Polaroid plates. The two layers are clearly differentiated, the lower one showing double refraction and the upper one being isotropic. The only treatment this protein received was storage for several weeks at 0°. Fig. 7 is a photograph of the same tube taken through "parallel" Polaroid plates. Bawden and Pirie (2) found the bottom layer to contain the higher concentration of protein and obtained no significant difference in infectivity of the two layers as measured on a protein basis. They described the bottom layer as consisting of "a three-dimensional mosaic of regions arranged at random to each other, but in each of which all the rod-shaped particles will lie approximately parallel" (2). We have found that if a small quantity of bottom layer material is removed, placed on a slide, covered with a cover-slip, and examined with a polarizing microscope, the field appears to be a two-dimensional mosaic of doubly refracting areas oriented in different directions. If one rotates the slide through 45°, areas previously dark appear light, and conversely. This is typical double refraction. Top layer material, which does not exhibit spontaneous double refraction, shows no double refraction when examined in a similar manner with the polarizing microscope. Bawden and Pirie (2) made essentially the same observation as that just described, and apparently this is their reason for considering the bottom layer a three-dimensional mosaic. If this is a true picture of the condition of the bottom layer, it is entirely analogous to a suspension of doubly refracting crystals of microscopic size in an isotropic medium. We actually find that, as one rotates the polarizer and crossed analyzer about the beam of light passing through the bottom layer, no change in light

intensity detectable to the eye is observed. The bottom layer as a whole, then, exhibits a special type of double refraction, inasmuch as it has no extinction directions.

It is interesting to observe that a suspension of small tobacco mosaic virus protein crystals contained in a test-tube shows the same behavior between crossed Polaroid plates as the bottom layer just described. Furthermore, ordinary solutions of tobacco mosaic virus protein, which do not show spontaneous double refraction in the unaltered state (see Figs. 6 and 7, top layer), do show this same special type of double refraction when acidified to the isoelectric range or when treated with 10 per cent  $(\text{NH}_4)_2\text{SO}_4$  and acetic acid. All of this may be interpreted to mean that the crystals of the tobacco mosaic virus protein are in themselves doubly refracting. However, this reveals nothing about the state of refraction of the virus protein molecules in solution. No evidence has been obtained from these studies which would allow one to decide whether the native virus protein molecules are isotropic or anisotropic.

The interpretation of the negative results obtained in the cases of the protein of the Shope papilloma virus and the elementary bodies of vaccinia must be considered. Negative results mean that the double refraction of the system is either zero or some value too small to be detected by the method employed. This may obtain with particles which are not sufficiently asymmetrical to be oriented appreciably in a viscous stream in which the shear is small. The elementary bodies, which are quite large, have been photographed and appear to be fairly symmetrical. Very small particles, on the other hand, regardless of the degree of asymmetry, can be oriented only by very strong mechanical fields because of the tendency to become randomized again owing to Brownian movement. Another possibility, however, exists which would account for low or unmeasurable values of stream double refraction. A consideration of Wiener's theory reveals that in the case of isotropic asymmetrical particles, low or even undetectable values of double refraction of flow will obtain if the index of refraction of the particles approaches that of the suspension medium. Failure to detect double refraction of flow in solutions or suspensions cannot be regarded as proof that the suspended particles or dissolved molecules are symmetrical.



## SUMMARY

A micromethod for the quantitative comparison of the double refraction of flow of different materials has been described. Purified proteins isolated from tobacco plants infected with the following diseases were found to show the phenomenon of double refraction of flow: tobacco mosaic, aucuba mosaic of tomato, severe etch, tobacco ring spot, latent mosaic of potato, Holmes' masked tobacco mosaic, and a mild tobacco mosaic. Elementary bodies of vaccinia and the Shope papilloma virus protein were not found to show stream double refraction. *Limulus* and *Helix* hemocyanin and hog thyroglobulin were found to show a small amount of double refraction of flow when concentrated solutions were used.

The double refraction of flow in tobacco mosaic virus protein solutions appears to be due to the orientation of rods rather than to the photoelastic effect, for the phenomenon is observed in solutions of relatively low viscosity, and there is no direct relationship between change in viscosity and change in stream double refraction. Furthermore, a time lag in the disappearance of double refraction was observed after the cessation of streaming, especially in systems near the isoelectric point. Double refraction was also found to persist after a stream of virus solution was allowed to pass from a pipette into the air, thus showing that the double refraction is not directly dependent upon shear, and hence that it is not due to the photoelastic effect.

Upon standing, relatively concentrated solutions of tobacco mosaic virus protein were found to separate into two layers, the bottom one of which is spontaneously doubly refracting. This confirms the observations previously reported by Bawden and Pirie.

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## THE INTERMEDIATE FORMS OF OXIDATION-REDUCTION OF THE FLAVINS

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### *Present Stage of Knowledge of Quinhydrone of Lactoflavin*

If the term quinhydrone is used only to characterize the level of oxidation, the details of the structure or molecular size being disregarded, a quinhydrone in the dissolved state may be said to be capable of existence at least in two forms, either as a semiquinone radical, or as a valence-saturated dimeric compound. In a series of former papers concerned with a great variety of dyestuffs, only the radical could be shown to exist. In a recent paper, however, with a suitable dyestuff as a model (1), the co-existence of the two forms in equilibrium could be demonstrated. The proof depended on the comparison of the titration curves obtained with varied concentrations of the dye. These observations challenge a reinvestigation of the nature of the quinhydrone of the flavin dyestuffs and especially lactoflavin, which has been shown capable of existence, in a previous paper (2). In this the quinhydrone was tacitly assumed to be a radical, in analogy to the many other cases investigated before. The experience with the phenanthrenequinone dye in the paper just mentioned makes a reinvestigation urgent. The task is to reinvestigate the shape of the titration curve at varied concentrations. The slight solubility of these dyestuffs seemed to make this task difficult. We have found, however, that accurate titration curves can be obtained even at very low concentrations of the dye and that it is therefore possible to vary the concentration widely in spite of the small solubility of the dye. These experiments will be presented in this paper.

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Another reason for a reinvestigation of the problem is an observation by Kuhn and Ströbele (3), concerning the multiplicity of the quinhydrone-like compounds in the flavin dyestuffs in the crystalline state, which will be discussed later on.

### *Experiments*

The overcoming of the difficulty ensuing from the slight solubility of these substances, especially in the reduced state, is due to the following favorable properties of these dyes. They have the faculty of establishing quickly constant and reproducible potentials even at extremely low concentrations. Especially with lactoflavin the experiments could be carried out in solutions differing in concentration in the ratio of 20:1. In fact, our lowest concentration used is by far not that lower limit which would just allow the establishment of well reproducible potentials. The practical limit is dictated only by the fact that the determination of the zero and end-points of titration can no longer be estimated with the desired precision, if the concentration is too low. The electromotive activity in the better poised region is quite remarkably great even at concentrations of  $10^{-5}$  mole per liter.

In the second place, since even the highest concentrations of the dye used in these experiments is very small ( $5.4 \times 10^{-4}$  M), all difficulties concerned with maintenance of pH and ionic strength during any one titration are automatically eliminated, whereas in the case of phenanthrenequinone-3-sulfonate, the overcoming of these difficulties was a substantial part of the technique. A third fortunate circumstance is this: All changes which could be expected from a variation of concentration were manifested within this very low concentration range obtainable. This was likewise not the case with the phenanthrene dye, in which the influence of concentration would have been entirely overlooked if we had been confined to such a low range of concentration.

All experiments were reductive titrations with hydrosulfite, with the technique essentially as described before. To check the accuracy of the method, a series of titrations with the same technique was performed with a number of freshly prepared perfectly pure dyestuffs of the indophenol group, oxidative and reductive titrations (although not with hydrosulfite which would not be permissible with indophenols), about which we shall report on

another occasion. In all these control experiments the index potentials varied, at 30°, from 14.4 to 14.6 millivolts. Leaving it open for the time being, whether the discrepancy from the expected value 14.3 is real or within the limits of error, we can at least say that the uncertainty amounts at most to 0.2 millivolt and usually less.

The experiments were executed with glucoalloxazine and with riboflavin (lactoflavin), with the same samples synthesized in this laboratory as used for the previous experiments. One set of experiments was made at pH 4.62 (acetate buffer), another at pH 6.92 (phosphate buffer). Here the constancy and reproducibility of the potentials are amazingly perfect. A third was attempted at pH 9 (veronal buffer). However, here the potentials were not perfectly stable. The drift was always towards the positive side. It was so slight that it would have been of minor importance for ordinary purposes. But it seemed risky to use these experiments for the calculations to follow in which an error of a few tenths of a millivolt entails noticeable consequences. In the experiments at lower pH all potentials were reproducible, with two platinum electrodes in the same vessel within less than 0.1 millivolt and in duplicate experiments to at least 0.2 millivolt and usually even better. The change of pH due to the process of reduction is entirely negligible in the concentrations used. No other uncontrollable factor which might influence the pH was present. This was ascertained in some cases by measurements of pH of the solution before and after the titration experiment with the glass electrode. The change in total volume during the titration never exceeded 4 per cent.

The experiments for riboflavin at pH 6.92 are plotted in Fig. 1 without any correction whatever. The symmetry around the mid-point is not strictly perfect. This must be attributed mainly to the uncertainty in the determination of the true zero point. This uncertainty is about 2 per cent and negligible for ordinary purposes; *e.g.*, for the determination of the normal potentials or index potentials with a claim of accuracy within  $\pm 0.2$  millivolt. But for the potentials of the first quarter of the curve it may cause a distinct error. It would have been easy to apply small corrections by changing the zero point very slightly. Since such a procedure would be quite arbitrary, no corrections were applied

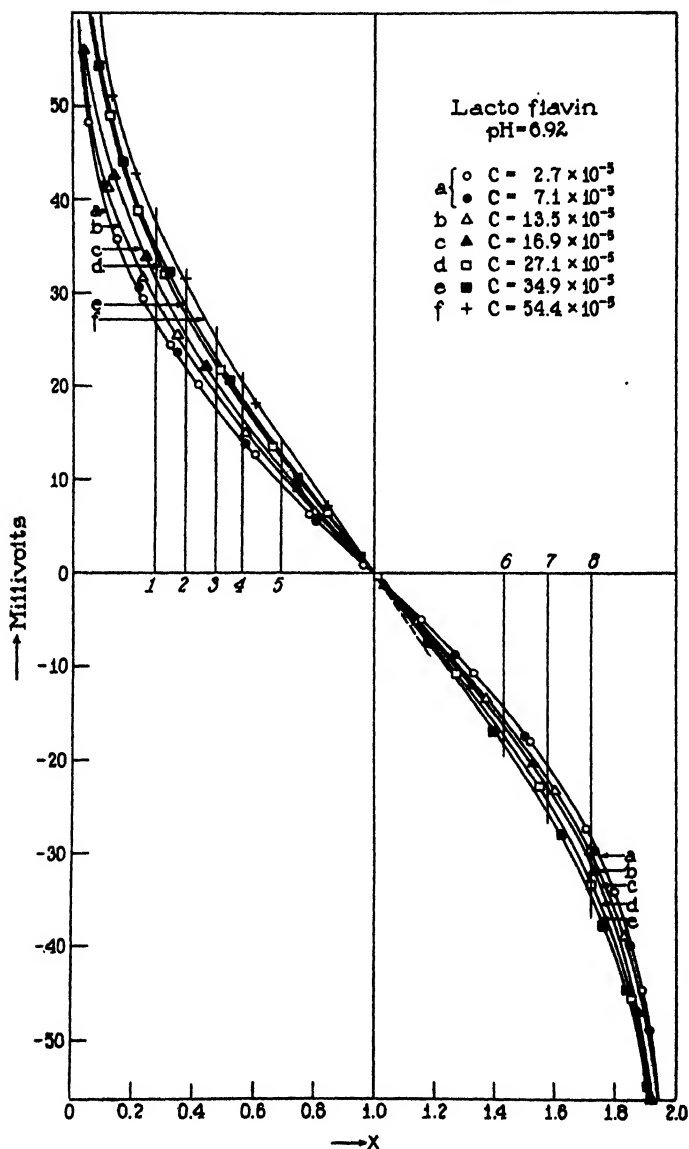


Fig. 1. Potential of lactoflavin for varied initial concentrations at pH 6.92, plotted against equivalents of added reducing agent. The normal potential is taken as zero point ( $30^\circ$ ).

at all. The error involved in this small inaccuracy was recognized by not utilizing for the calculations of the constants the part of the curves too close to the zero point. The reason for the error in the zero point lies in the method employed consisting in titrating out the oxygen. The ideal method would be to wash out the oxygen completely with nitrogen. On working with those low concentrations of the dye any trace of oxygen left would greatly interfere and its complete removal of bubbling with  $N_2$  would take an unduly long time and yet be unsafe. The uncertainty of the end-point, however, is much smaller and entirely negligible.

TABLE I  
*Glucisoalloxazine at pH 6.87 and 4.62, at 30.0°*

pH	Concentration of dye	Index potential		$E_m$
		$x = 0.5$	$x = 1.5$	
6.87	$\times 10^{-3} M$			
	4.5	15.2	15.2	-0.1595
	9.9	15.0	15.2	-0.1595
	12.7	15.2	15.2	-0.1593
	19.7	15.2	15.2	-0.1600
	20.8	15.15	15.2	-0.1955
	41.2	15.7	15.7	-0.1950
4.62	41.2	15.7	15.7	-0.1955
	9.5	15.2	15.1	-0.0522
	21.0	15.0	15.3	-0.0516
	40.2	15.3	15.6	-0.0514

The results for glucisoalloxazine are given in Table I. The potential at 50 per cent reduction (when  $x = 1$ ) is used as zero point for each curve.

#### *Calculation of Constants*

*Glucisoalloxazine*—It can be seen that in the case of glucisoalloxazine at pH 6.87, from the lowest concentration almost up to the highest, the index potentials have the constant value of 15.2 millivolts. For glucisoalloxazine the quinhydrone is therefore present entirely in the radical form. Only at the highest concentration can an indication for dimerization be found. From



the index potential,  $E_i = 15.2$  millivolts, of the curves obtained at the lower concentrations we obtained the semiquinone formation constant  $k$ .

$$\frac{s^2}{r \times t} = k = (A - (3/A))^2 = 0.0147$$

where  $A = 10^{\frac{E_i}{0.06}}$  and  $s$ ,  $r$ , and  $t$  are the molar concentrations of the semiquinone, the fully reduced, and the fully oxidized forms. As maximum ratio of semiquinone to total dye we find

$$(s/a)_{\max.} = \frac{\sqrt{k}}{2 + \sqrt{k}} = 0.057$$

From the index potential,  $E_i = 15.7$ , at the highest concentration we can now calculate the dimerization constant

$$\gamma = \frac{d}{s^2} = 1.75 \times 10^4$$

The method of this calculation will be presented presently.

The value of  $\gamma$  can be, of course, only approximate. A small change in the index potential causes a great alteration. The constant is given only to get an idea about the order of magnitude for comparison with the constant for riboflavin, which will be calculated with greater precision.

*Riboflavin (Lactoflavin)*—The reduced form of riboflavin has at pH 6.92 a solubility which is sufficient to reach a total concentration of  $5.4 \times 10^{-4}$  M. This is higher than was anticipated to be possible, since the reduced form always appeared very little soluble. The reason for the increased solubility of the reduced form is probably that, at this pH, the reduced form is present in two forms of acidic ionization in equilibrium with each other, on account of its  $pK = 6.2$ , according to the previous paper. This should increase the solubility. At this pH, all potentials are of an ideal constancy and reproducibility, except for the second half of the titration curve (Curve *f*, Fig. 1), for the highest concentration, where precipitation of the reduced form begins. For this particular experiment, Fig. 1 shows only the first, reliable half of the curve.

The experiments at the two lowest concentrations yield entirely

coinciding curves. This shows that we have reached at these concentrations the limiting curve for infinite dilution, with an index potential of 16.8 millivolts.

The curves for higher concentrations are distinctly displaced, and sufficiently so as to allow the calculation of the dimer dissociation constant. The theory of this calculation will be given in a way adapted to the situation, and somewhat different from the previous one (1).

We have used, so far, only the index potential for the calculation of  $k$ . Since this value is a fundamental one for the calculation of the other constant, it is desirable to check it by using not only the potential at 25 per cent (or 75 per cent) reduction, as is done in the index potential method, but also other points of the curve. This can be done as follows: Let  $x$  be the equivalent amount of the reducing agent used per mole of the dye; then  $x$  varies during the titration from 0 to 2. Let  $t$ ,  $s$ ,  $r$  be the concentrations of the totally oxidized, the semiquinone, and the reduced form and  $a$  that of the total dye. Then we have for any point of the limiting curve

$$r + s + t = a \quad (1)$$

$$s + 2r = xa \quad (2)$$

$$\log \frac{t}{r} = \frac{E}{0.030} \quad (3)$$

where  $E$  is the potential, referred to potential at  $x = 1$  as zero point.  $E$  is positive in the first half and negative in the second half of this reductive titration. From these equations we obtain

$$r = a \times \frac{(1-x)}{(N-1)}$$

$$t = a \times \frac{N(1-x)}{(N-1)}$$

$$s = a \times \frac{x(N+1)-2}{(N-1)}$$

where  $N = 10^{\frac{E}{0.03}}$ . This gives all the data to calculate  $k = s^2/rt$  for any point of the curve. The calculation in Table II has been carried out for eight points.

Proceeding now to the curves at higher concentration, we start with the assumption that the displacement is due to the formation of a dimeric compound. Let  $d$  be its concentration. Then we have for any point

$$r + s + 2d + t = a$$

$$s + 2d + 2r = xa$$

$$\log \frac{t}{r} = \frac{E}{0.03}$$

$$\frac{s^2}{rt} = k = 0.117$$

From these equations one obtains

$$r = a \times \left( \frac{1-x}{N-1} \right)$$

$$t = a \times N \times \left( \frac{1-x}{N-1} \right)$$

$$s = a \times \sqrt{kN} \times \left( \frac{1-x}{N-1} \right)$$

$$d = \frac{a}{2} \times \left( 1 - \frac{(1-x)(1+N+\sqrt{kN})}{N-1} \right)$$

where  $N = 10^{\frac{E}{0.03}}$ . These data are sufficient to calculate  $\gamma = d/s^2$  from any point of the curve. If we arrive at the same value of  $\gamma$  irrespective of the point chosen, and of the particular curve, this is sufficient evidence that the distortion of these curves is really due only to the appearance of the dimeric form, and that no other molecular species of the kind to be discussed later on is formed in these solutions to any noticeable amount.

The dimeric compound may be also considered as a compound of 1 molecule of the totally oxidized form, and 1 molecule of the reduced form. The constant of the equilibrium is

$$q = \frac{d}{rt} = \gamma k$$

the dimeric formation constant.

For the calculation of the mean value of  $k$  the figures from the



points 1 and 2 (Fig. 1) have been neglected, because, as has been mentioned before, the sources of error are much larger at the beginning of the curves. For the same reason the points 1 and 2 have not been used for the calculation of  $\gamma$ . In the calculation of the mean value of  $\gamma$  the figures derived from different curves have been given different weights: the value from Curve *f*, the weight 5; from Curve *e*, 4; Curve *d*, 3; Curve *c*, 2; Curve *b*, the weight 1. This is justified, because the values become, with increasing concentration and increasing index potential, more and more accurate, as the figures show clearly. It is quite natural that

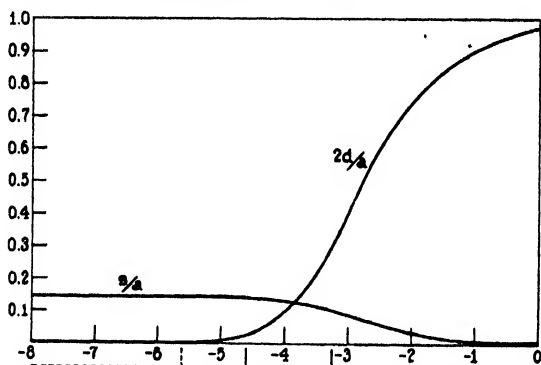


FIG. 2. The maximum ratio of semiquinone to total dye ( $s/a$ ), and of dimeric form to total dye ( $2d/a$ ), plotted against the logarithm of the total concentration, for lactoflavin at pH 6.92. The bracket at the abscissa indicates the experimental concentration range; the dotted bracket, the physiologically occurring one.

Curve *b* yields the lowest values for  $\gamma$ . This is the effect of the assumption that the concentration of the dimeric form in Curve *a* is already low enough to consider it as infinitely small, which is, of course, only an approximation.

From these data the diagram (Fig. 2) has been constructed, showing the ratio in which the dye exists in the semiquinone form,  $s/a$ ; and the ratio in which the dye exists in the dimeric form,  $2d/a$ , in the mid-point of titration (at  $x = 1$ ) where the concentration of the intermediate forms is maximal. For this calculation we start from any arbitrary value of  $r$ ; this equals  $t$ ; furthermore,  $s$  and  $d$  are calculated with the values of the two equilibrium

constants calculated above; then  $r + t + s + 2d = a$ . The latter is plotted logarithmically.

Another set of experiments has been carried out at pH 4.62. Here the reduced form of riboflavin is very sparingly soluble. The maximum concentration at which the titration can be carried out is 3 mg. in 50 cc. ( $1.2 \times 10^{-4}$  M). Up to this concentration the variation of concentration causes no definite change of the

TABLE III  
*Riboflavin at pH 4.62, at 30.0°*

Concentration of dye  $\times 10^{-4}$ M	Index potentials		$E_m$
	$x = 0.5$	$x = 1.5$	
2.0	15.7	15.9	-0.0968
3.1	15.8	16.2	-0.0970
4.9	15.5	15.5	-0.0958
7.4	16.0	16.3	-0.0968
12.3	15.9	16.1	-0.0960
18.5	16.7		-0.0960

index potential, as shown in Table III, the mean value being 16.0 millivolts. Herefrom we obtain

$$k = 0.0504$$

$$(s/a)_{\max.} = 0.10$$

#### *Improvement of Diagram of Three Normal Potentials*

In the previous paper, the diagram of the three normal potentials plotted against pH was derived from the assumption that the semiquinone formation constant was practically alike over the pH range from about 3 to at least 10. This constant was derived from an average value of the index potentials obtained over this whole pH range. Since we have now reached such a degree of accuracy that we need no longer rely on such an average value, we can improve this diagram.

We start from the following argument. The semiquinone formation constant,  $k$ , and the difference of the normal potential of the higher step and that of the lower step,  $E_1 - E_2$ , are as follows:

pH.....	4.62	6.92
$k$ .....	0.0504	0.117
$E_1 - E_2, mv$ .....	98.0	55.1

Thus we have two points for the  $E_1$  and  $E_2$  curves, one at pH 4.62, the other for pH 6.92. These two points are the basis for the construction of the diagram (Fig. 3) with the principles de-

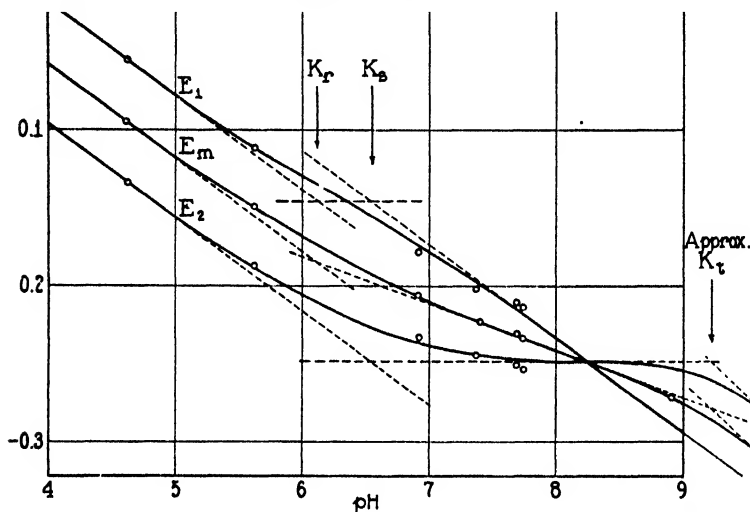


FIG. 3. The three normal potentials of lactoflavin plotted against pH, at 30°.  $E_1$  normal potential of the system reduced + semioxidized,  $E_2$  that of the system semioxidized + totally oxidized form,  $E_m$  that of the system reduced + totally oxidized form.  $k_r$ ,  $k_s$ ,  $k_t$ , bends due to ionization constants of the three forms. The location of  $k_t$  is only approximate because of the drifts of the potentials in this pH region. The small deviation of the points obtained for  $E_1$  and  $E_2$  at pH 7.9 from the theoretical, drawn out curves is accounted for in the text.

veloped previously. It differs somewhat from the previous diagram which was constructed from an average value of  $E_1$ . The  $pK$  of the semiquinone has been displaced, from 8.0 to 6.5. This causes convergence of the  $E_1$  and  $E_2$  curves with increasing pH, resulting in a crossing with the  $E_m$  curve at pH 8.2. It is now our task to check these curves interpolated and extrapolated from those two points experimentally for other points. For this reason further titrations were performed at such a low concen-

tration of the dye as to obtain the limiting curve for infinite dilution. These experiments were perfectly satisfactory and reproducible at pH 5.6 and 7.4. They yielded the points for  $E_1$  and  $E_2$  given in the diagram. At pH 7.70 and 7.75 the potentials were no longer strictly constant but showed a very slight drift in time, always towards the positive side. The drift was so slight that its influence on the normal potential,  $E_m$ , is not noticeable in a plot of the scale used, but it is liable to cause a slight error in  $E_i$ . Owing to the drift, the whole curve is compressed along the potential axis. The observed  $E_i$  becomes too small, and the distance from  $E_1$  to  $E_2$  becomes too great. All these errors are very small. In fact, the distance  $E_1 - E_2$ , as calculated from the uncorrected index potential, is just a bit greater than the theoretical plotting requires. The direction of this very slight but unmistakable error is in the expected direction. At higher pH the drift becomes greater and makes any accurate calculation useless. Nevertheless, at pH 9.0, where the drift was already quite appreciable, the index potential, as read from the uncorrected curve, was 18.6, and we can be sure that the necessary correction would amount to quite a number of millivolts; the value expected from Fig. 3 is 23 millivolts. This is at least compatible with the extrapolated curve.

The potential drift in the alkaline region is obviously due to some irreversible alteration of the dye. It is not an effect of light, since all titrations were performed in the dark. The direction of the drift suggests that it is always due to the destruction of the reduced form. It is probably the destruction by alkali of the alloxan ring in the reduced form. It should be noticed that the pH limit for the perfect stability coincides with the pH limit in the living organism of higher animals, pH 7.5.

From the data obtained we can calculate the maximum ratio of semiquinone to total dye at various pH values as follows (for 30°):

pH.....	5	6	7	8	9
$(s/a)_{\max}.....$	0.10	0.10	0.14	0.28	0.50

For the physiological pH range, these values are distinctly higher than those given previously. It is a peculiar situation that a dye of the phenazine type, showing, of course, a wide separation of



the two steps of oxidation in very acid solution, should have, first in a very acid solution, and again in a very alkaline solution, such a wide separation of the steps. This is due to acidic dissociation in the alloxan ring.

*Discussion of Influence of Conditions Other Than in Experiments Described*

If lactoflavin is not in homogeneous solution or if it is in combination with the specific protein, the situation is different from that in our experiments. Let us consider first the crystalline state. This is, if at all, comparable more to a highly concentrated solution than to a diluted one. So a high degree of dimerization may be expected in the crystals. In fact, Kuhn and Ströbele found a paramagnetic susceptibility of less than one-tenth that expected for a free radical on measuring the crystalline chloroflavin, which is on the oxidation level of the semiquinone, or its dimeric compound. For the crystalline state it may not be appropriate to speak of a mixture of semiquinone and its dimer, but rather of a state intermediate between the two forms, a resonance between each pair of adjacent molecules in the crystal grating such that the molecular unit resembles a bimolecular more than a unimolecular structure. Such an idea is quite compatible with the fact that the definition of molecular unit in the crystalline state is ambiguous anyway.

Kuhn's verdoflavin, in contrast, which is supposed to be a compound of  $1S + 1T$ ,<sup>1</sup> has a magnetic susceptibility half that of a free radical. These crystals may be said to consist of alternate units of a radical and of the oxidized form, and adjacent molecules will resonate between these two forms. So we may imagine a compound of 1 molecule of  $S$  with 1 molecule of  $T$  to be the molecular unit. This contains in any case 1 odd electron, and should show just the magnetic susceptibility observed by Kuhn and Ströbele.

These authors compare the number of subdivisions in the oxidation levels of lactoflavin with that of hemoglobin with its 4 Fe atoms. This comparison does not seem significant, even

<sup>1</sup> The reduced, semioxidized, and totally oxidized forms, each in its simplest unimolecular form, are designated as  $R$ ,  $S$ , and  $T$ .

though the fact that the one-fourth and the three-fourths quinhydrones do not exist in solution be disregarded. For Kuhn himself assumes dissociation of these compounds in solution, and the arguments presented in the present paper rule out the existence of any other compound than *R*, *T*, *S*, and *D* in solution. But even if there are compounds of all these intermediate levels, none of them can operate as an oxidizing or reducing agent of a valency less than 1. No molecule can detach or attach less than 1 whole electron. In the case of a quadrinuclear iron complex the situation is different, as has been recently shown by Michaelis and Smythe (4). Here the 4 Fe atoms can each detach or attach a single electron in succession. Kuhn's observations of the various intermediate states in the crystalline state will, however, keep its significance for the problem of the structure of crystalline quinhydrones, for which ratios of the quinoid and the hydroquinoid forms different from 1:1 have been found on several occasions; for instance, 1:2 in a crystalline preparation of Wurster's blue (Willstätter and Piccard (5)), or 2:5 in a crystalline quinhydrone of *p*-phenylenediamine + benzoquinone (Schlenk (6)). There is no evidence for their existence in solution.

Proceeding to the combined state as it exists in the flavin-phosphate-protein compound, we can infer nothing from our investigations. All constants may be considerably shifted. Haas (7) has shown that the yellow enzyme, when reduced by triphosphopyridine nucleotide (coenzyme), passes in a nearly neutral solution through a red intermediate state with the same absorption spectrum as the radical obtainable with the free lactoflavin only in extremely acid solution. If this be so, we may conclude that the semiquinone radical formation constant is greater for the flavin-enzyme than for the free lactoflavin, and that the electronic structure of the radical in combination with the protein and the coenzyme resembles that of the free radical in extremely acid solution, where it is in combination with a proton. This consideration may give us the clue as to the place where the protein or the coenzyme is attached. Such a state of affairs is quite in line with the knowledge about the changes of characteristic properties of one and the same porphyrin compound according to whether it is free or combined with the one or the other specific protein.

## SUMMARY

The reductive titration curves for some alloxazine dyes, glucoisalloxazine and especially riboflavin (lactoflavin), have been reinvestigated, for varied pH values and for varied concentrations of the dye. An analysis of the titration curves reveals that at low concentrations, including the concentration range occurring under physiological conditions, there is an intermediate form of reduction which is entirely represented by a free radical. The maximum ratio of this to the total dye is 0.10 at pH 4.62, and 0.14 at pH 6.92, at 30°. In higher concentrations of the dye a partial dimerization of the radical to a bimolecular compound takes place, which can be accurately measured for lactoflavin at pH 6.92. The equilibrium constant of this dimerization is calculated. No other molecular species on an oxidation level between flavin and dihydroflavin can be detected in solution. On the basis of the experimental data the diagram of the three normal potentials against pH has been improved, as compared with that given previously. The conditions in the crystalline state, or in combination with the specific proteins, are different from those in a simple and homogeneous aqueous solution of the dye and are discussed.

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## PROPERTIES OF HEMOGLOBIN AND PEPSIN IN SOLUTIONS OF UREA AND OTHER AMIDES\*

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The occurrence of manyfold increases in the solubility of both native and denatured proteins in the presence of certain specific dissolved substances has been known since the observations of Spiro (53), Ramsden (49), and others. These large increases in solubility have received no satisfactory explanation, but the lytic effect of one of the most effective of these substances, urea, has been utilized in dissolving, or keeping in solution, denatured proteins (8), and for investigating the solution properties of proteins of low solubility (19, 31, 72). However, Burk and Greenberg (15) and Burk (13), who measured the osmotic pressures of several proteins at high concentrations by dissolving them in 6.66 *M* urea, deduced that while the molecular weight of ovalbumin was the same in the mixed solvent as in water the molecular weights of a number of others, including horse hemoglobin, were halved or even further lowered. The change in molecular weight for horse hemoglobin was confirmed by Wu and Yang (72), who showed that ox hemoglobin also dissociates in urea, but that the hemoglobins of sheep and dog do not. Similar changes in molecular weight in urea solutions have been described for myosin (70). Burk has pointed to the consistency of these observations with protein dissociations which occur outside the "pH stability regions" in the ultracentrifugal investigations of Svedberg and his collaborators.

\* A preliminary account of this work appeared in *Nature* (54).

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Burk and Greenberg likewise observed that their proteins became insoluble after removal of the urea, and, in the case of hemoglobin, suffered changes in the characteristic light absorption. This observation appeared consistent with an earlier report (7) that this protein was denatured by concentrated urea. Wu and Yang also described their proteins as denatured, and concluded from the species differences observed that splitting and denaturation, though both may be brought about by urea, are distinct phenomena.

The great chemical diversity in the substances influencing protein solubility suggests that their effects are not all of the same kind. Among them, *glycerol* and *phenol* are protein solvents even when unmixed with water; the latter was used in attempted protein molecular weight determinations even before urea-water mixtures (67, 25, 18). A third liquid, *formamide*, shares with *urea* its capacity to induce denaturation under certain conditions and, like urea, has a much greater effect on solubility than does glycerol or phenol, when all three are compared in low concentrations. *Sodium salicylate* denatures at least one protein, methemoglobin (9), and is likewise effective as a small mole fraction of the total solvent. The more moderate effect of *amino acids* can be quantitatively ascribed to the large dielectric constant increment which they contribute to the medium (33, 73, 17).

The powerful solvent action of urea is shared by other amides, of which formamide, mentioned above, is only a single example.<sup>1</sup> Almost equally great effects (on the solubility of crystalline pepsin) have been observed by the author with other substances containing an amide bond, acetamide and urethane.<sup>2</sup> It has been shown (30) that exposure of ovalbumin and serum proteins to substances containing an amide bond, in which 1 hydrogen atom on the nitrogen is unsubstituted, always produces characteristic changes in the reactivity of the protein sulfur. These changes are also

<sup>1</sup> It has been reported that urea increases the solubility of starch. Addition compounds with a large variety of both inorganic and organic substances are known. In the present paper, urea is regarded as an amide on the basis of modern theories of valency and evidence from Raman spectra (personal communication from Dr. John T. Edsall).

<sup>2</sup> These solubility measurements are to be reported in another paper.

produced by all treatments which denature (23, 29). When both protein and amide are present in high concentrations, striking changes in physical properties—sharp increases in viscosity, precipitation on standing, or irreversible setting to a hard plastic form—are often observed. After removal of the amide by dialysis or dilution, denatured protein precipitates even when the amide solution has remained clear. These observations, and others typical of denaturation (10), parallel so closely the effects of amides on solubility that a causal connection has come to be assumed. Anson and Mirsky's equilibrium theory of denaturation (9) explicitly correlates the two phenomena by finding, in these effects on solubility, evidence for changes in activity coefficients which bring about a shift in a postulated equilibrium between native and denatured forms. The mechanism of the inordinately large solubility or activity effect is itself left unexplained.

The author's observation of the effect of urea and other amides on the solubility of the crystalline protein, pepsin, draws attention to the need for such an explanation, because here the phenomenon is uncomplicated by any possibility of denaturation. The specific activity of the enzyme, stored and measured in urea concentrations in which it is 10 times more soluble than in water, is quantitatively unchanged. Enzymic proteins are well suited for studies of this kind because they are characterized, in the native state, by highly specific kinetic properties, easily measured in the media under examination. In previous investigations the most common criterion of denaturation has been loss of solubility at the isoelectric point after the denaturing agent (which also keeps the denatured protein in solution) has been reduced in concentration by dilution or dialysis. This criterion is unavoidably ambiguous, since it is impossible to distinguish between denaturation produced by the high concentration directly and denaturation brought about at the later stage of removal of the urea. In certain cases, as with hemoglobin and serum albumin, there is no doubt that the protein has undergone a distinct change while still in the urea, although, as will be shown here, it is not the effect usually called denaturation. In the case of pepsin the activity is unchanged while in urea and acetamide, and, under

certain conditions, even after subsequent precipitation.<sup>3</sup> Recently it has been shown (14) that with this protein exposure to urea causes no increase in the nitroprusside reaction. Nevertheless, true denaturation of pepsin is readily produced by alkali or by heat, and very much higher concentrations of urea are required to bring this truly denatured inactive material into solution than were required to dissolve the active material originally. There is thus little reason for thinking of this protein as denatured while initially in urea.

Because of these differences in the effect of urea on the dissociation, denaturation, and solubility of different proteins, it seemed very desirable to study the effect of amides on another protein with readily measured specific properties. This requirement is fulfilled by horse hemoglobin: its capacity to combine reversibly with oxygen and carbon monoxide is lost on denaturation, and a large literature describes transformations, characteristic of either the native or denatured state, which it can be made to undergo and which are readily followed by observations of the corresponding changes in absorption spectra. The osmotic pressure measurements on hemoglobin by Burk and Greenberg and Wu and Yang, the experimental basis of which has been criticized by Hand (22), furnished another incentive for the selection of this protein, since the results of these authors might indicate that the changes in solubility are merely one manifestation of molecular dissociation or of the forces inducing it. At the outset, therefore, measurements were made with both hemoglobin and pepsin by the ultracentrifugal sedimentation velocity method of Svedberg, and the diffusion methods of Lamm and Polson (37), with a number of other amides as well as urea.

#### EXPERIMENTAL

*Hemoglobin*—Corpuscles were separated by centrifugation from freshly drawn defibrinated horse blood saturated with carbon monoxide. After repeated washing with CO-saturated 0.9 per

<sup>3</sup> The author has crystallized pepsin directly out of acetamide solutions. Anson and Mirsky have observed that mixtures of active and inactive trypsin show the proper activity in urea solutions. However, they believe that urea prevents the reversal of inactivation of part, which would otherwise occur.

cent NaCl, they were stirred with 3 volumes of distilled water and 1 volume of toluene, and left in the ice chest overnight. The hemoglobin solution, practically free of stromata, was decanted from under the toluene and electrodialed against cold distilled water for 3 hours, at temperatures below 15°. During this process considerable precipitation occurred as salt was removed. The resulting solution was filtered, resaturated with CO, and stored at 3-5° in small, steamed out glass-stoppered bottles filled to overflowing. Dry weight determinations indicated a hemoglobin content of 3.6 to 3.7 per cent;<sup>4</sup> the conductivity of the stock solutions was approximately  $2 \times 10^{-5}$  reciprocal ohms. Spectroscopic examination showed normal CO bands which were converted to oxyhemoglobin bands by prolonged exposure to air in thin layers.

Stock solutions, when kept cold, saturated with CO, in unopened bottles, remained unchanged for weeks. Bottles which had been repeatedly exposed to air but resaturated with gas sometimes contained a slight sediment, and showed the presence of a small amount of aggregated material in the sedimentation velocity diagram. These bottles were discarded. No detectable trace of methemoglobin appeared in any of the material used. Thanks are due to Mr. K. Andersson for one of the two stock solutions used in these experiments.

Before use, water and recrystallized KCl were added to the stock solution to make the final concentration of salt 0.1 M (in two runs this was increased to 0.25 M). The final concentration of protein was varied according to the thickness of the centrifuge cell (2 or 3 mm.), between 0.45 and 0.65 gm. per 100 cc. The diluted solutions, resaturated with CO, were used either immediately after mixing, or after storage at temperatures between 0-25° for intervals of time up to 2 weeks. When amides were used, solutions made up gravimetrically, to the same concentrations of protein and salt for measurements of partial specific volumes, were usually used in the other work. Owing to the requirement of sufficient refractive power, only the higher of the

<sup>4</sup> This concentration is considerably higher than would appear possible from an extrapolation of A. A. Green's measurements of the solubility of hemoglobin at low salt concentrations; but it has been confirmed repeatedly.



protein concentrations were used in diffusion measurements. The same protein and salt concentrations were used for the spectroscopic observations and gas-combining capacity measurements as in the measurements of molecular weight. This is a necessary limitation, since it must be expected that a dissociation equilibrium will be a function of concentration as well as of medium; observations by Tiselius and Gross (66) and by Pedersen and Andersson (44) indicate that hemoglobin is appreciably dissociated in simple salt solutions at concentrations of protein below 0.4 per cent. The upper limit of concentration is fixed by the great light absorption of the protein at 546  $m\mu$ , the wavelength used. The presence of salt is required to minimize the Donnan effect (65); the adequacy of the amount used is shown by the experiments with more than twice the usual concentration.

Buffers were avoided because use of high concentrations of amides would require taking into account shifts in buffer equilibria caused by changes in the medium (Burk and Greenberg described such shifts, and attempted to allow for them). Hemoglobin in equilibrium with buffer at its isoelectric point in water may interact with the buffer in 40 per cent urea, if the buffer acid undergoes a change in strength in the new medium. In the absence of buffers, pure uncombined protein must remain uncombined and electrically neutral, since it is unlikely that the relative strengths of the carboxyl and imino or amino groups could be changed sufficiently for internal proton migration to occur. The narrow width of the pH stability region of this protein, with the isoelectric point narrowly inside its acid limit although near neutrality, made this precaution particularly desirable. pH measurement with both hydrogen and glass electrodes (found, pH 6.9 to 7.0) showed that electrodialysis had brought the protein solution close to the isoelectric point. In concentrated amide solutions the pH can be given no precise significance, but the potentials found never differed by more than 4 millivolts from those obtained in simple salt solutions. The case of formamide is an exception to this statement and is discussed elsewhere.

*Pepsin*—Three times crystallized enzyme was prepared according to Philpot's method (46), and dissolved to saturation at 22° in 0.1 M KCl containing 0.002 M HCl. Repeated small portions of solvent were used with an excess of crystals, the first two por-

tions being discarded. A practically constant solubility of 2.0 gm. of pepsin per liter was obtained for several successive equilibrations, the activity of diluted aliquots being used as the method of analysis (55). On 4-fold dilution with solvent, sufficient light absorption remained for use in the centrifuge with a 12 mm. cell.

These solutions were stored at 3° until used (1 to 15 days) with no appreciable loss of activity. The sedimentation velocity diagram showed that 20 per cent of the light absorption was caused by relatively non-centrifugable material. This fraction, previously noted by Philpot, corresponds to the appreciable amount of "non-protein nitrogen" which is found in all such acid pepsin solutions, and which increases only very slowly in the cold (55). Most of the runs were made with diluted stock solutions dialyzed in the cold in narrow cellophane tubing for 2 days against 0.1 M KCl, 0.002 M HCl. The non-centrifugable light absorption decreased, but a part, from 6 to 14 per cent, remained.

The final concentration of enzyme varied between 0.05 and 0.12 gm. per 100 cc., the higher concentrations being used in a 6 mm. cell. When high concentrations of amides were used, dialyzed stock solution was added directly to the weighed out amide without further addition of water. In such cases the concentration of salt and acid was lowered by about one-fifth.

As with hemoglobin, buffers were dispensed with; the 0.002 M acid kept the solution near the isoelectric point of pepsin at approximately pH 2.7.

*Urea, Acetamide, Formamide, Glycine*—c. p. urea was twice dissolved in 70 per cent ethanol at temperatures under 40° and twice recovered at -9°. The crystals were washed with absolute alcohol and air-dried at 50°. The conductivity (1 M solution,  $5.1 \times 10^{-6}$ ; 4 M,  $6.3 \times 10^{-6}$  at 20°) was only slightly greater than that of the distilled water, and considerably lower than that of Burk and Greenberg's material. Tests for acid or base combination were negative.

Schering-Kahlbaum's acetamide was dissolved at 35° in 80 per cent of its weight of methanol. After the solution was filtered and cooled to below room temperature, 5 cc. of ethyl ether per gm. of acetamide were added. The crystals were redissolved in methanol at 35° and filtered at -9° after the addition of 10 volumes of anhydrous ether. The crystals were air-dried, vacuum-desic-

cated over sulfuric acid, and finally warmed to 60° in an oven for a few hours. They were stored over  $P_2O_5$ . A molar solution was faintly acid to brom-thymol blue, but its conductivity was only slightly higher than that of the distilled water.

Merck's formamide was purified by the method of Verhoek (68), with a distillation temperature 10° higher than Verhoek's and a slightly less pure distillate. Only one fractional crystallization was carried out. The material obtained melted at 2.15° (m.p. of purest formamide 2.55°), and had a conductivity of  $1.1 \times 10^{-4}$  which doubled during the period of storage before use (a noticeable odor of HCN also developed). A 10 per cent aqueous solution containing hemoglobin and salt (Experiment 48) had a pH of 6.18 measured by a glass electrode, indicating some decomposition into acid products. The material was stored frozen in a dry atmosphere.

De Haen's glycine "*nach Sørensen*" was used without further purification.

*Sedimentation Velocity Measurements*—Sedimentation constants were measured according to the current practise in the Upsala laboratory (58, 59), the light absorption method being used to record the sedimentation of the protein. With hemoglobin the mercury green line (546 m $\mu$ ) was isolated by a Wratten No. 77 filter and photographed through the rotating cell on Ilford Chromatic plates. With pepsin, Ilford process plates and the usual chlorine and bromine filters for the ultraviolet were substituted. Permanent concentration standards of *o*-cresolphthalein were used for hemoglobin, and potassium dichromate for pepsin. Speeds of 54,000 to 70,000 R.P.M., corresponding to forces between 210,000 and 340,000 times gravity, were employed. Eight to ten exposures were used in calculating the rate of sedimentation in experiments running between 90 and 240 minutes, according to the viscosity of the solution and the sedimentation rate. The temperature rose gradually in each run; average temperatures are given in Tables I and III. All sedimentation constants are expressed as their equivalents in pure water at 20°, by correcting for the viscosity of the solutions and the change in density and viscosity of water with temperature, according to the usual equation:

$$s_{20}^{\circ} \text{ (corrected)} = \frac{\frac{\delta x}{\delta t}}{\omega^2 x} \left( \frac{\eta_{t^{\circ}}}{\eta_{20}^{\circ}} \right)_{\text{H}_2\text{O}} \cdot \frac{\eta}{\eta_0} \cdot \frac{1/V - \rho_0}{1/V - \rho} \cdot \left( \frac{1 - V\rho_{20}^{\circ}}{1 - V\rho_{t^{\circ}}} \right)_{\text{H}_2\text{O}} \quad (1)$$

$s_{20}^{\circ}$

in which

- $\eta$  = viscosity of solvent
- $\rho$  = density of solvent
- $\eta_0$  = viscosity of water
- $\rho_0$  = density of water
- $V$  = partial specific volume of protein
- $\omega$  = angular velocity
- $x$  = distance of boundary from the center of rotation

These correction factors assume unusual importance in the present experiments which involve very high concentrations of dissolved substances. Their adequacy is attested by experiments on hemocyanin dissolved in deuterium oxide (61) and by the results with pepsin reported in this paper. Viscosities and densities have been taken from the International Critical Tables, interpolating where necessary. The density used for the glycine solution was measured.

*Diffusion Measurements*—The refractometric method of Lamm and Polson (37) was employed, with red light and sensitized Cramer process plates. Diffusion constants were calculated by two of the methods described by these authors (Equations 2 and 3 of their paper) from at least three exposures spaced over about 30 hours; the values given in Table II are based on Equation 2, since the values of  $\sigma$  calculated from the coordinates of the entire curve are almost identical with directly measured values of  $\mu$  used here. Values of the diffusion constant calculated from  $\sigma$  (Equation 3) usually agree with those in Table II within 1 per cent.

The homogeneity of the protein in the various solvents was tested by fitting the experimental points with the normal curve, as described by Lamm and Polson. The author wishes to thank Mr. Alfred Polson for much help with these measurements and for making the actual exposures.

*Partial Specific Volumes*—Hemoglobin was added to solvent by weighing out stock solution, allowance being made for the water added by omitting an equivalent amount in the final dilution.

At the same time other portions of stock solution were weighed out for dry weight analyses. At least two such sets of pycnometric determinations at 20° were made in each solvent.

Since no significant changes in the specific volume of hemoglobin were found in the mixed solvents, the corresponding quantity for pepsin was not measured; Philpot's value in aqueous salt solutions was employed for this protein throughout.

*Pepsin Activity*—A slight modification of the hemoglobin-tyrosine method of Anson and Mirsky was employed (55). Test solutions of hemoglobin contained the same concentration of urea or acetamide as the enzyme solution. These substances are without effect on the amount of color produced by digestion products with the Folin reagent, but their presence greatly increases the blank of chromogenic material unprecipitated in 0.15 M trichloroacetic acid.

*Absorption Spectra*—Band positions were measured with a Hartridge reversion spectroscope. Thanks are due to Dr. G. Millikan of the Physiological Laboratory at Cambridge for a calibration of the instrument and for valuable guidance in making the measurements. Detailed examination of the CO hemoglobin bands was made with a Koenig-Martens spectrophotometer. In the conversion of hemoglobin into various derivatives (details given in Table IV) care was taken to keep the amide concentration nearly unchanged.

*Gas Capacities and Affinities*—Oxygen- and carbon monoxide-combining capacities were measured through the kindness of Major J. H. C. Walker at Cambridge. Barcroft tonometers and the Van Slyke constant volume manometric technique (45) were employed. The ratio of the affinity of hemoglobin for the two gases was measured by comparing the apparent position of the mixed absorption bands in water and in urea solution, after long equilibration with CO-air mixtures of widely different known compositions, as in the method of Hartridge.

### *Results of Measurements*

Table I summarizes the sedimentation constants for the two proteins in all the experimental solutions. The column at the extreme right gives the corrected constant adjusted for all differences due solely to changes in density and viscosity, as described in the preceding pages. Residual differences are therefore due to

changes in the proteins themselves—either in state of dispersion, hydration, or in various other factors which may affect their frictional coefficients.

With hemoglobin, these differences are well marked and vary in a regular way. The control runs in aqueous salt solutions give values near  $4.6 \times 10^{-13}$  for this protein, in good agreement with the value (4.5) usually given. With concentrations of urea up to 4 M, and with acetamide concentrations up to 6.46 M, the sedimentation constant falls regularly with increasing concentrations, reaching a limiting value of 3.1 or 3.2 in solutions of both substances. Higher concentrations of urea—almost double those at which this value is first reached—do not lower the sedimentation constant further. A maximum effect has already been produced.<sup>5</sup>

The lowest concentrations of formamide employed (2.26 M, 10 per cent) are also apparently sufficient to produce the same limiting effect. With higher concentrations, however, the constant falls slightly; at the same time the protein becomes unstable, turning brown within a week at room temperature. Diffusion data at this concentration also show anomalies. At still higher concentrations (9.38 M, 40 per cent) the protein is moderately stable in the cold, but its color changes rapidly at higher temperatures, its absorption for the mercury green line almost vanishing during the course of a centrifuge run. The sedimentation constant rises. Possibly the trace of acid impurity present in these formamide solutions is responsible for these secondary effects.

It should be noticed that an almost saturated solution of an

<sup>5</sup> The continuous variation of an apparent sedimentation constant requires some explanation. With the resolving power at present available with the light absorption method used here mixtures of molecules with small sedimentation constants will not show two clear inflection points in the sedimentation velocity diagram because of the relatively rapid rate at which these smaller molecules diffuse. In place of two normal S-shaped curves in the graph relating protein concentration to distance from the center of rotation, one long curve with an anomalously shallow slope is found. With larger molecules discrete curves can be separated, but in the present case it is expedient to treat the curve as single; the intermediate value of the sedimentation constant must be considered as that of a weighted average of the two molecular species present. Complete resolution of these molecular species is possible when Lamm's refractive index method is substituted for light absorption.

TABLE I  
Sedimentation Constants

Experi- ment No.	Solvents		Time of wait	Tem- pera- ture during wait	Protein con- centra- tion	Aver- age temper- ature of run	$s_{20}^0$ $\times 10^{13}$	Cor- rection factor	$s_{20}^0$ (cor- rected) $\times 10^{13}$
(a) Hemoglobin									
Practically no dissociation									
	moles			$^{\circ}\text{C.}$	gm. per 100 cc.	$^{\circ}\text{C.}$			
1		Water	No wait		0.63	31.7	4.48	1.030	4.61
30		"	" "		0.65	30.4	4.55	1.030	4.68
35		"	" "		0.58	33.3	4.56	1.031	4.70
36		"	" "		0.44	25.9	4.45	1.019	4.54
37		"	" "		0.36	33.3	4.51	1.030	4.65
46		"	" "		0.47	33.0	4.49	1.028	4.61
Average								4.63	
Partial dissociation									
2	1	Urea	3 hrs.	5	0.64	31.9	3.80	1.128	4.29
4	1	"	6 days	5	0.64	35.4	4.06	1.129	4.59
13	1	"	5 "	20	0.64	30.5	3.98	1.113	4.44
39	2.96	"	3 "	5	0.43	33.3	2.93	1.389	4.06
21	1	Acetamide	4 hrs.	5	0.65	30.4	3.52	1.199	4.22
22	1	"	3 days	20	0.65	30.4	3.76	1.200	4.51
23	3.04	"	2 hrs.	20	0.65	32.3	2.69	1.501	4.04
24	3.04	"	3 days	20	0.65	33.4	2.71	1.507	4.09
26	4.49	"	1 hr.	20	0.65	30.5	1.92	1.812	3.48
29	4.49	"	5 days	5	0.50	31.1	1.93	1.803	3.48
51	2	Glycine	2 hrs.	20	0.48	34.0	2.44	1.780	4.34
Practically total dissociation									
3	4	Urea	3 hrs.	5	0.64	32.4	2.08	1.572	3.27
5	4	"	4 days	5	0.64	32.0	1.99	1.573	3.13
17	4	"	1 day	20	0.66	27.2	2.07	1.537	3.18
18	4	"	4 days	20	0.66	27.3	2.11	1.534	3.23
11	6.48	"	1 day	5	0.47	30.8	1.52	2.120	3.22
15	6.48	"	8 days	20	0.47	28.6	1.52	2.115	3.21
9	7.46	"	2 hrs.	5	0.50	29.2	1.37	2.422	3.32
31	6.46	Acetamide	2 "	20	0.64	32.0	1.43	2.362	3.37
33	6.46	"	1 day	20	0.55	31.3	1.36	2.361	3.21
40	6.46	"	4 days	20	0.41	34.2	1.48	2.363	3.50
48	2.26	Formamide	No wait		0.47	30.2	3.10	1.133	3.40
50	2.26 0.25	" KCl	1 hr.	20	0.47	36.4	2.83	1.159	3.28

TABLE I—*Concluded*

Experi- ment No.	Solvents		Time of wait	Tem- pera- ture during wait	Protein con- centra- tion	Aver- age temper- ature of run	$\eta_{30^{\circ}}$ $\times 10^{13}$	Cor- rection factor	$\eta_{30^{\circ}}$ (cor- rected) $\times 10^{13}$
Grossly unstable or inhomogeneous									
	<i>moles</i>			$^{\circ}\text{C.}$	<i>gm. per 100 cc.</i>	$^{\circ}\text{C.}$			
42	4.55	Formamide	1 day	5	0.50	33.3	2.34	1.248	2.92
43	4.55	"	3 days	20	0.50	31.8	2.40	1.258	3.02
44	9.38	"	2 "	5	0.50	32.0	2.34*	1.602	3.75*
47	9.38	"	6 "	5	0.50	32.4	2.59*	1.602	4.15*

(b) Pepsin  
No dissociation

53		Water			0.05	26.9	2.83	1.024	2.90
54		"			0.05†	26.7	2.93	1.024	3.00
57		"			0.06	27.1	3.08	1.024	3.15
58		"			0.14†	27.6	3.04	1.026	3.11
55	1	Urea	3 hrs.	20	0.06	26.8	3.05	1.118	3.41
59	4	"	2 "	5	0.10†	27.5	2.20	1.536	3.38
60	6.46	Acetamide	8 "	20	0.08†	27.9	1.41	2.341	3.30

All solvents contain 0.1 M KCl, except in Experiment 50. Protein concentrations in a few cases discussed in the text appeared to diminish slightly on waiting long periods, and are therefore lower than the figures given. Viscosity corrections used with *acetamide* are based on the viscosity of acetamide solutions at 25°, corrected for the change of viscosity of *water* between this temperature and the temperature given. This simplification gives corrections which may be wrong by a few per cent.

\* These figures are very uncertain owing to bleaching of the protein (with respect to the wave-length used) during the run.

† Before dialyzing; final solutions slightly more dilute.

amino acid (2 M glycine) shows only a barely significant effect. Table I also shows that the fall in sedimentation constant to the level characteristic of each solution occurs in a very short time, in at most an hour at centrifuge temperatures. Further changes do not occur, either in the cold or on storage at room temperature. Secondary changes in formamide solutions which are exceptions to this statement have already been mentioned. With high concentrations of acetamide, long storage at room temperature caused gradual formation of a cloudy suspension; similar phenomena have been observed by others with dilute ovalbumin



solutions in urea. The results tabulated are not complicated by the consequences of this secondary change in stability (never observed with urea), and reflect only establishment of an initial equilibrium, which is very rapidly attained.

Pepsin, unlike hemoglobin, is not affected by high concentrations of urea and acetamide. The molecular weight of the enzyme is half that of the respiratory protein (47), with a value which has been shown to be the most common basic protein unit (57, 60, 59).<sup>6</sup> The absence of any decrease in the *corrected* constant for pepsin in the solvents which produced the maximum effect with hemoglobin attests the legitimacy and precision of the large corrections which have been applied in the two cases. Likewise, it tends to eliminate the possibility of other more obscure interfering medium effects. Further support for this conclusion is contributed by the fact already noted that with hemoglobin the sedimentation constant, which falls progressively in the range of urea concentrations between 0 and 4 M, shows no further fall when very much larger correction factors are used with concentrations almost twice as high.

*Diffusion Constants*—The results of four diffusion experiments are summarized in Table II. All three exposures in the sets with 4 M urea and with 6.46 M acetamide give self-consistent values of *D*; the averages of both sets, when corrected for differences in viscosity, are almost identical: 7.81 and  $7.68 \times 10^{-7}$  respectively. These are very near the well established value for ovalbumin (7.75), and larger than that for normal horse hemoglobin, 6.3 (66). Since the sedimentation constant in these two media is *lower* than that of ovalbumin, the molecular weight of hemoglobin in these two solutions cannot be greater than that of the latter protein dissolved in water.

With 1 M urea the constants are less consistent, with their average nearer to the normal value. The downward drift indicates that more than one molecular species is present, a result already forecast by centrifuge experiments in the same solution. Progressive dissociation by dilution at the boundary may con-

<sup>6</sup> Further interest attaches to the observation that urea-treated pepsin also differs from most proteins in not giving an increased nitroprusside reaction (14).

tribute to the result.<sup>7</sup> Application of graphical tests for homogeneity to the results with both urea concentrations showed no

TABLE II  
*Diffusion Constants (D) of Carboxyhemoglobin in Amide Solutions  
Containing 0.1 M KCl*

The data for 4 M urea and 6.46 M acetamide show total dissociation, and satisfy tests for homogeneity.

Solvent		Protein concentration	Time	$\mu^*$	$\mu$ calculated†	$D_{20}^\circ \times 10^7$	Viscosity correction	$D_{20}^\circ$ (corrected) $\times 10^7$
moles		gm. per 100 cc.	hrs.					
1	Urea	0.67	13	2.43	2.50‡	7.64	1.039	7.93
			21	2.91		6.82		7.08
			36	3.84	4.05§	6.91		7.19
4	Urea	0.66	12.5	2.14	2.14	6.40	1.215	7.76
			19.5	2.70	2.73	6.49		7.89
			25.5	3.07	3.08	6.40		7.78
						Average		7.81
6.46	Acetamide	0.72	12	1.605		3.70	2.102	7.79
			18	1.94		3.61		7.59
			24	2.25		3.64		7.66
						Average		7.68
4.55	Formamide	0.72	7.03	2.30		8.00	1.112	9.79
			19.08	3.50		6.85		8.39
			25.00	3.87	3.28	6.39		7.81

\* Measured distance between inflection points.

† Calculated from moments of the entire curve unless noted.

‡ The ratio of area to maximum height gives 2.46.

§ The ratio of area to maximum height gives 4.00.

|| This figure was obtained from the ratio of area to maximum height.

significant deviations from the normal curve. However, with the more dilute solution, the value of  $\mu$ , calculated arithmetically

<sup>7</sup> It seems likely that the values obtained for hemoglobin in ordinary salt solutions may be slightly high for the same reason: at any concentration, diffusion of the smaller particles (always in equilibrium with larger ones) will be faster.

from the moments of the experimental curve, differs appreciably from the value calculated from the height and area on the assumption that the curve is normal. These minor discrepancies, sensitive indications of inhomogeneity, do not appear at the higher concentration, where dissociation is apparently nearly complete. The small scale line displacements (resulting from very low protein concentrations) do not permit the calculation of  $\mu$  from the area or from the computed moments of the curve except in a few favorable cases. Elsewhere  $\mu$  has been taken as half the distance between the two inflection points in the data, a normal curve being assumed and the exact position of the inflection determined by dividing the maximum height of the curve by  $\sqrt{e}$  to get its height at these points.

In the formamide experiment, the apparent diffusion constant decreased with time and was anomalously high. Partial dissociation into particles of less than half normal size is indicated. In keeping with this conclusion, a serious discrepancy appears between  $\mu$  measured off from the distance between the inflection points and  $\mu$  calculated from the height and area or from the moments of the 25 hour curve. Graphical comparison shows an appreciable deviation from the ordinates of the normal curve.

Since the area of each curve in any one series was invariant and closely followed the protein concentration, it is concluded that the refractive power of the protein in these solutions underwent no change.

*Molecular Weight*—It has been shown (36) that Svedberg's equation relating molecular weight to sedimentation and diffusion constants

$$M = \frac{RTs}{D(1 - V\rho)} \quad (2)$$

in which  $R$  is the gas constant,  $T$  the absolute temperature,  $D$  the diffusion constant,  $s$  the corrected sedimentation constant, and the other symbols have the meanings assigned in Equation 1, rests on the same assumptions as the sedimentation-equilibrium equation, which may be derived thermodynamically.<sup>8</sup> If

\* Lamm's derivation is strictly valid only for systems of two components. When more are present, as in these experiments, a condition for its validity is that no combination occurs between protein and any component, as

$3.15 \times 10^{-13}$  is used as the value of  $s$ , and  $7.80 \times 10^{-7}$  as the value of  $D$ , for hemoglobin in concentrated urea solutions,  $M$  is almost exactly 39,000. The normal value of  $V$  (0.749 at  $20^\circ$ ) has been employed in this calculation, since direct determination in these solutions scattered within 1 per cent of this value.

With 6.46  $M$  acetamide, both  $s$  and  $D$  show that the protein is slightly less dissociated; the average molecular weight is therefore a little higher. In both cases, it is slightly greater than half the value of  $M$  assigned to horse hemoglobin (67,000) as the average obtained by various methods of measurement (1, 60), in agreement with accurate analytical data for human hemoglobin (41). In view of the constancy of  $s$  at urea concentrations between 4 and 8  $M$ , it seems unlikely that the failure to give the half value exactly is caused by incomplete dissociation. Errors in  $M$  ascribable to uncertainties in  $V$  (about 2 per cent),  $s$  (3 per cent), and  $D$  (1.5 per cent) are insufficient to account for it. Viscosity corrections make up the greatest part of the corrections applied to  $s$  and  $D$ ; these cancel out almost entirely in computing  $M$ , and cannot be at fault (the results with pepsin indicate this also). There is a still greater discrepancy (in the same direction) between the molecular weight of ovalbumin as calculated from sedimentation and diffusion (60) and the weight inferred from measurements of osmotic pressure and from analytical data. These discrepancies, of obscure origin, have only secondary importance for the purpose of this research.

These findings confirm by a wholly independent method the conclusions of Burk and Greenberg, based on their measurements of osmotic pressure. The substantial deviations from ideal osmotic behavior which they found were met by introducing large empirical correction terms, which were interpreted as an allowance for hydration of the protein. This interpretation was carried further by Hand (22), who denied that dissociation was required to explain their data. He used viscosity measurements of concentrated hemoglobin solutions in urea to show that the abnormally high osmotic pressure in urea is due to a virtual increase in protein concentration through removal of water as water of

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Adair has pointed out (2). It is assumed that no serious discrepancies arise from this source in isoelectric dilute salt solutions, where the major extra component is electrically neutral.

hydration, rather than to molecular splitting. Hand made use of the equation of Kunitz (34, 35) to calculate the change in hydration of the protein induced by urea, and concluded that the change was sufficient to account for the observed increase in pressure. This conclusion is disputed here for two reasons: first, it is inconceivable that the low protein concentrations of the present experiments can bind enough water to account for any considerable osmotic effect; and second, it is unlikely that any extensive change in hydration could be without measurable effect on the apparent partial specific volume, as measured in this investigation. Since the present experiments confirm those of Burk and Greenberg, Hand's criticism of their experiments appears to be without foundation. It is not denied that changes in hydration may accompany the long time secondary changes in viscosity in urea solutions purposely avoided in these measurements (they occur much more rapidly with oxyhemoglobin than with carboxyhemoglobin), but they are probably smaller than postulated by Hand. Other factors than hydration (change of shape, dissociation) may affect the frictional constant of a labile dissolved substance, and may consequently affect the viscosity of its solution. The enormous hydration postulated by Hand seems very excessive in view of a number of careful hydration determinations which agree in finding that proteins combine with less than one-third of their weight of water (62, 5, 3). The empirical correction term of Burk and Greenberg (2.8 gm. of water per gm. of protein) is thus too large to be interpreted as hydration but the Kunitz equation would indicate still greater effects,—over 35 molecules of water per amino acid residue, in the case of gelatin. Its use for calculating hydration from viscosity has been criticized in detail by Adair and Callow (4).

*Reversal of Dissociation*—Sedimentation measurements were carried out on dialyzed hemoglobin solutions which had contained various concentrations of amides for different periods of time, in order to ascertain whether dissociation is reversible. Dialysis was carried out in the cold in cellophane bags, against five to twelve changes of 0.1 M KCl.

In these experiments (Table III)  $s$  increases to values only slightly lower than those for normal hemoglobin; at the same time the inhomogeneity of the protein increases. In several runs material with sedimentation constants considerably larger than

TABLE III  
Sedimentation Constants in Dialyzed Solutions

Ex- peri- ment No.	Solvent	Time of ex- posure	Tem- pera- ture of ex- posure	Time of dialysis	Aver- age temper- ature of run	$s_{20} \times 10^{13}$	$s_{20}^{\circ}$ (cor- rected) $\times 10^{13}$	
Partial dissociation before dialysis								
	moles	days	°C.	days	°C.			
6	1	Urea	6	5	3	31.1	4.5*	4.5*
7	1	"	6	5	3 + 4	31.1	3.92†	4.02†
14	1	"	7	5	2	29.7	5.04‡	5.16‡
16	1	"	7	5	2 + 7	31.5	5.15§	5.27§
41	2.96	"	3	5	3	32.1	4.27	4.37
25	1	Acetamide	4	5	4	29.8	4.09	4.20
27	1	"	4	5	4 + 4	29.1	4.17	4.36
28	3.04	"	4	20	6	29.9	4.14	4.25
32	3.04	"	4	20	6 + 3	31.6	4.18	4.30
34	4.49	"	7	5	3	31.6	3.95	4.05
Total dissociation before dialysis								
	4	Urea	3	5	4	32.2	3.91¶	4.04¶
12	4	"	3	5	4 + 11	29.7	4.18	4.43
19	4	"	4	20	Graduated steps	31.5	3.77¶	3.87¶
20	4	"	4	20	" " + 8	32.2	3.95	4.06
38	6.46	Acetamide	10	20	3	33.5	4.00	4.07
52	2.26	Formamide	2	5	2	32.9	3.92**	4.04**
Grossly unstable or inhomogeneous before dialysis								
45	4.55	Formamide	12	5	2	32.0	9.0***	9.0***
49	9.38	"	8	5	2	34.2	No analysis possible due to rapid change dur- ing run	

All solutions contained 0.1 M KCl. The symbol + indicates the time of storage after dialysis when a second run was made at a later date. During this period a slight decrease in concentration occurred, manifested by loss of light-absorbing capacity and precipitation. Where this is considerable or where serious inhomogeneity developed, it is indicated by notes.

\* One component only. The solution showed several inflection points, the others referring to heavier components.

† Homogeneity increased.

‡ 15 per cent loss in light absorption; inhomogeneous even before dialysis.

§ Increase in heavy components.

|| 25 per cent loss in light absorption; almost homogeneous.

¶ Loss due to precipitation as described in text; remainder almost homogeneous.

\*\* Apparent loss, but color changing.

that of normal hemoglobin could be detected, although not readily measured. Occasionally this material sedimented only slightly more rapidly than the remainder and is averaged in with the mixture of dissociated and apparently reversibly associated molecules (Experiments 14 and 16), but usually part of this rapidly sedimenting material is separable and is not included in the constant given. This tendency of dialyzed dissociated hemoglobin to excessive aggregation is also shown by the slight rise in  $\alpha$  which always occurs on storage of dialyzed solutions. Sedimentation constants of previously *totally* dissociated solutions tend to be slightly lower just after dialysis than those of solutions previously only partly dissociated. On storage for a week or longer, however, both approach the normal value, in spite of peculiarities described below. The proportion of anomalously aggregated material also increases, and a small quantity of protein may precipitate. The duration of exposure to the amide solution before dialysis is apparently not a determining factor.

Totally dissociated solutions are sharply distinguished from all others by the occurrence, during dialysis, of irreversible precipitation of a definite fraction of the total protein. The size of this fraction appears to depend on the amide concentration. With 4 M urea, it is about 30 per cent, and increases to well over half at 6.48 M; with 7.46 M hardly any color remains in the dialyzed solution. With 6.46 M acetamide about 25 per cent precipitates. No precipitation occurred on dialysis of formamide solutions, but these solutions had undergone other changes.

Variation in the manner of carrying out the dialysis is without influence on the size of the precipitated fraction. With 4 M urea, precipitation occurred when the urea concentration was reduced below 2 M, regardless of whether dialysis went rapidly (1 hour against 0.1 M KCl) or very slowly (several days, against graded steps of diminishing urea concentrations). These regularities, ordinarily suggestive of the existence of equilibria, remain obscure. It will be shown that the protein in the urea solution prior to dialysis cannot be distinguished into two fractions on the basis of detectable differences in specific properties; further, it is difficult to account for these precipitations by postulating the existence of a slowly labile equilibrium with urea, because the *rate* of dialysis in such a system should largely determine the proportion of material

precipitating: with sufficiently slow dialysis, little should remain to precipitate. The changes which induce precipitation may therefore occur only on removal of the dispersive influence of the urea. Other phenomena consistent with this view are presented below; the occurrence of total precipitation on dialysis or dilution of very concentrated urea protein solutions has contributed much to the current opinion that the protein is denatured in these solutions.

*Relation of Dissociation to Denaturation*—Hemoglobin which has undergone *dissociation* in amide solutions has not simultaneously suffered the loss of specific properties, which accompanies *denaturation*. These properties include the positions of its absorption bands and the bands of its common derivatives, its combining capacity for oxygen and carbon monoxide, and the relative affinities of the protein for these gases at different partial pressures.

Absorption band positions of some of the simpler derivatives in urea and formamide and methods of their formation are summarized in Table IV. No significant differences between the band positions in water or in the mixed solvents have been detected.<sup>9</sup> Each derivative may be formed in more than one way, and the transformations may be reversed a number of times. The characteristic reduction and oxygenation cycle, and others involving the replacement of oxygen by carbon monoxide, hemochromogen formation, and methemoglobin, may be directly observed spectroscopically in amide solutions. Detailed comparison of the structure of the CO bands in water and in 4 M urea with a Koenig-Martens spectrophotometer showed their complete identity; on reexamination a week later, only a barely detectable diminution in the intensity of absorption in the urea solution had occurred. Hemoglobin in dialyzed urea solutions also appeared normal if care had been taken to exclude oxygen at every stage in its handling.<sup>10</sup>

A single difference was observed between the aqueous and amide solutions. Methemoglobin, formed on dissolving a crystal of

<sup>9</sup> Small shifts of purely optical origin might well have been expected in such concentrated solutions.

<sup>10</sup> Pedersen has shown that very dilute hemoglobin solutions are *partially* dissociated, but Ray and Blair (50) found that dilute hemoglobin solutions exhibit no spectroscopic anomalies.



TABLE IV

*Summary of Band Positions of Hemoglobin Derivatives*

The figures given are in Ångström units and represent averages of several measurements with different preparations. They are significant to 3 Å, except for certain diffuse bands. The figures in bold-faced type represent intense bands; those in parentheses weak or diffuse bands. All solutions contained 0.7 per cent hemoglobin and 0.1 M KCl.

	Solvent		
	Water	25 per cent urea (4.4 M)	20 per cent formamide (4.5 M)
Carboxyhemoglobin	5695	5698	5703
	5365	5373	5375
Oxyhemoglobin (a)	5767	5767	5767
	5409	5408	5405
Methemoglobin (b)	6307	6303	6303
	(4860)	(5350)	
		(4970)	(4990)
Alkaline hemochromogen (c)	<b>5579</b>	<b>5583</b>	<b>5583</b> (NH <sub>3</sub> present)
	5280	5280	5278
Carboxyhemochromogen (d)	5700	5707	5701 (NH <sub>3</sub> present)
	5374	5376	5383
Reduced hemoglobin (e)	(5560)	(5558)	(5558)
"Parahematin" (f)		(5341)	(5350)

*Methods of Formation*—(a) Prolonged exposure to air in thin layers, with occasional stirring, for a day. Most of the transformation from the CO compound occurs in the 1st hour. The slight amount of mechanical denaturation resulting from stirring causes a little precipitation in the aqueous solution; the dispersive effect of urea and formamide prevents its manifestation in the others. (b) A very small crystal of potassium ferricyanide in 1 cc. of solution produces an effect within an hour. With the amide solutions, in which further changes take place, the measurements were made after 2 hours. (c) Solid NaOH and Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> were added to oxyhemoglobin. Alkali by itself fades the bands, and the solution becomes brown. Carboxyhemoglobin behaves similarly but more slowly. Solutions saturated with CO appear to undergo no transformation when treated with both alkali and hydrosulfite, owing to the formation of CO hemochromogen, indistinguishable from CO hemoglobin (38, 52). (d) CO added to hemochromogen (c); also produced when CO hemoglobin is treated by alkali, or alkali and hydrosulfite; it may be distinguished from CO hemoglobin by its failure to transform to methemoglobin on treatment with ferricyanide. (e) Oxyhemoglobin was exposed to a vacuum for several minutes, or reduced with hydrosulfite. Reoxygenation to about 80 per cent of capacity is possible when formed the first way; hydrosulfite-treated solutions may be reoxygenated, but are almost instantly transformed to methemoglobin. (f) Methemoglobin was allowed to stand for 10 to 20 hours at 25° in solutions of urea or formamide. After longer periods other changes ensue. These are probably the bands observed by Burk and Greenberg (see text).

potassium ferricyanide in the amide solutions, is less stable than when formed in pure water. Within 20 hours at 20–25° the characteristic band in the red fades out and is replaced by a diffuse band in the green (about 534 m $\mu$ ). This new band is characteristic of parahematin ("cathemoglobin"), and was observed by Burk and Greenberg in old solutions of oxyhemoglobin in 6.66 M urea. Parahematin has been identified as the denatured form of the *ferri* state of hemoglobin (32), and Burk and Greenberg concluded that half weight hemoglobin is denatured. It seems more probable that its formation is a secondary phenomenon, occurring only when formation of methemoglobin as an intermediate is possible. It has never been observed with carboxyhemoglobin.

This parahematin, contrary to the belief of Burk and Greenberg, is not identical with the substance studied by von Klaverin and by Keilin. The latter when reduced by sodium hydrosulfite in neutral or slightly alkaline solutions gives the unmistakable hemochromogen spectrum of reduced denatured hemoglobin. Protein denatured by mechanical shaking or by standing in urea for long periods at temperatures over 30° will give this reaction. The parahematin described above, however, shows no sign of the intense hemochromogen line on treatment with hydrosulfite. If the reductant is added gradually, the first effect observed is the reappearance of the methemoglobin red band. At this stage the iron is still trivalent; no combination with CO occurs. Addition of further reductant produces the diffuse absorption of *reduced hemoglobin*. If this reduced solution is quickly shaken with air, oxyhemoglobin bands reappear but methemoglobin reforms exceedingly rapidly in the presence of oxygen after hydrosulfite has once been used. This cycle has been repeated five times with a single solution by adding fresh hydrosulfite in minimal quantities. At this stage, addition of alkali with the reductant (to make the solution neutral) results in a strong hemochromogen spectrum which can be changed to that of carboxyhemochromogen by exposure to CO,—the protein is now almost totally denatured. The same result is obtained at once if the parahematin is allowed to stand in urea at room temperature for a few days before addition of the reductant, although the rather diffuse parahematin band does not undergo detectable change. The function of alkali in all these experiments is not, as in the direct formation of hemo-

chromogen, to denature the protein, but to shift the hemochromogen-forming equilibrium in the desired direction (28).

These observations suggest that consideration of spectroscopic evidence alone may fail to distinguish between denatured hemoglobin and hemoglobin that has gone one step toward denaturation. The existence of an intermediate stage between native and denatured protein, at which the process may still be turned either way, has often been suggested ("incipient denaturation" is Sørensen's phrase). The ambiguity of spectra as a criterion of denaturation has also been pointed out by Meldrum (38) as the result of a series of observations involving methemoglobin. Meldrum believed that his own experiments, and those of Anson and Mirsky on the "reversal of denaturation," could be explained as due to denaturation of a definite fraction of the protein, which then combines with the remainder, "protecting" it. It appears impossible to make use of Meldrum's concept in the present case. Because of the reappearance of methemoglobin on the first addition of reductant to "parahematin," a stage of oxidation (possibly of thiol groups) appears to be involved in formation of the intermediate. It is possible, however, that reduced hemoglobin is formed at once and that oxygen present in the solution causes immediate formation of methemoglobin except in the presence of excess reductant. This and other aspects of the anomalous parahematin require further study.

The results with methemoglobin show that urea may sometimes hasten the process of denaturation. Ovalbumin also denatures fairly rapidly in concentrated urea. In this respect, proteins seem to differ greatly, even those so closely related as carboxyhemoglobin and methemoglobin of the same species. These cases may explain the observations of flocculation, setting to hard gels, and increase in the nitroprusside reaction described by Hopkins and others, and the increase in viscosity of hemoglobin solutions reported by Anson and Mirsky (7) and Hand. In other cases, as with carboxyhemoglobin or pepsin, none of these phenomena appears for several weeks, nor can any loss of specific properties be detected. Thus, although denaturation *may* be hastened in certain cases by the presence of urea, this change is secondary and may appear long after molecular dissociation, which is complete, when it occurs, in much less than an hour.

As Wu and Yang's results with the hemoglobins of several species also indicate, the change in molecular weight is not itself denaturation.

The failure of high urea concentrations to denature hemoglobin is also demonstrated by measurements of its oxygen capacity after freeing it of CO by a day's exposure to air, in thin layers. The solutions, containing 7.54 gm. of hemoglobin per liter, should bind 0.45 mm of oxygen. This is barely more than double the amount in physical solution, 0.22 mm in water, 0.16 mm (estimated) in 25 per cent urea. The attainment of sufficient accuracy to make these comparisons significant with such dilute solutions therefore requires great care to avoid mechanical denaturation during the preliminary aeration. The smaller gas volume (0.5 cc.) of the Van Slyke apparatus must be used; with 2 cc. samples, a pressure difference due to total oxygen of about 4 cm. may be obtained.<sup>11</sup> With ordinary aqueous solutions, figures slightly below the theoretical capacity were obtained. In 25 per cent urea, total oxygen came to 0.59 mm per liter, which leaves 0.43 mm as the bound oxygen. Direct measurements of the CO capacity of freshly prepared 25 per cent urea hemoglobin solutions saturated with CO were also carried out. This procedure avoided all delays and extra handling of the protein, but involved the disadvantage of much larger corrections for dissolved gas. The control gave 10.9 cm. differential pressure, equivalent to 1.456 mm of CO per liter. Subtracting 1.019 for dissolved CO leaves approximately 0.44 mm, practically the theoretical capacity. The urea solution gave 1.213 mm of CO; from this was subtracted three-quarters of the previous figure for dissolved gas, leaving 0.45 mm. Essentially similar results were obtained with other sets of solutions. In view of the large corrections and potential sources of error, these agreements must be considered very satisfactory.

Horse myoglobin ( $s_{20}^0 = 2.04$ ;  $D_{20}^0 = 11.25$ ;  $M$  (average) = 17,350) (63, 64, 48) differs from hemoglobin in molecular weight, the position of its absorption bands, its rates of combination with oxygen and carbon monoxide, and in the equilibria governing both processes (64, 40, 26). The oxygen dissociation curve for myoglobin, which contains only one prosthetic group per mole-

<sup>11</sup> Tests showed that no ammonia was liberated from urea with the concentrations of alkali used, within the time of the measurements.

cule, is a simple rectangular hyperbola. Current theories attempting to explain the more complicated equilibrium for hemoglobin depend on its larger number of groups and, in certain instances, on their mutual interaction (20, 42, 21). This suggested the desirability of measuring the same function for the half weight protein.

Attempts to make such measurements in urea solutions were unsuccessful because evacuation of oxyhemoglobin in these solutions caused denaturation of part of the protein. Either reduced hemoglobin, like methemoglobin, is less stable in these solvents than in water, or the greater amount of bubbling that ensues during exhaustion, owing to the lower surface tension, caused denaturation at a rapid rate. It proved possible, however, to measure the *ratio* of the oxygen affinity to that for carbon monoxide. Mixtures of the two gases in known proportions were slowly bubbled through solutions of oxyhemoglobin, in water and in the mixed solvent, in front of the slit of the Hartridge spectroscope. The solutions were observed during several hours equilibration with mixtures resulting in apparent final band positions over the entire span. No difference was detected between the final mixed band positions in the two solvents.<sup>12</sup>

Amide-dissociated hemoglobin has thus lost none of its spectroscopic or physiologically significant properties; it still requires denaturation (by heat, shaking, or strong alkali) before giving the hemochromogen spectrum. Truly denatured material, which precipitates on dialysis, cannot be redissolved in urea concentrations which held it in solution before precipitation.

With *pepsin*, similarly, the enzymatic activity is not impaired by amides. This was tested as described in the experimental section. The trichloroacetic acid filtrate from a digest by the enzyme dissolved in aqueous 0.0005 M HCl gave a color with the phenol reagent equivalent to 0.0851 gm. of tyrosine per liter. The blank was 0.0089 gm., and the difference, representing the increase in non-precipitable chromogenic material, was therefore

<sup>12</sup> The difference in position between oxygen and carbon monoxide bands is correlated with the ratio of the corresponding affinities when the hemoglobins (and myoglobins) of different species are compared (6). Since neither band positions nor affinity ratio has changed, dissociated hemoglobin retains this relationship.

0.0762 gm. The corresponding filtrate from the same concentration of enzyme tested in 4 M urea (made up 3 hours before the test) gave color equivalent to 0.1195 gm. of tyrosine per liter, with a blank of 0.0301 gm. The increment was therefore 0.0894 gm., higher than the control rather than lower. Probably precipitation of *partly* split digestion products is less effective in urea solutions than in water. Repetitions with other urea concentrations after different waiting periods gave similar results; tests with casein as the test protein, with enzyme and protein both dissolved in acetamide, gave better correspondence. 10-fold dilution with water of more concentrated enzyme solutions just before the test minimized the effect of amide on the blank and caused all residual differences to disappear. Unless it is assumed that inactivation was reversed on dilution, it thus appears that pepsin, like hemoglobin, suffered no denaturation. In view of the approximate correspondence found with the undiluted solutions and the fact that inactive pepsin, precipitated at the isoelectric point, will not redissolve in 4 M urea, the alternative assumption of reversal of denaturation on dilution seems unnecessary.

#### DISCUSSION

Previous studies from the Upsala laboratory have shown that dissociation and aggregation of proteins are a wide-spread phenomenon. They commonly occur at the limits of a pH stability region, but changes in protein or salt concentrations are sometimes sufficient to bring them about,—as with thyroglobulin, the hemocyanins, and the serum proteins. In only a few instances have these changes been proved to be accompanied by irreversible denaturation.

Earlier centrifuge work with urea solutions has been limited to experiments on zein (69) which showed that its molecular weight is not changed.<sup>13</sup> The molecular dissociation of horse hemoglobin in amide solutions, demonstrated in the present paper, recalls the conclusion of Tiselius and Gross (66) that simple

<sup>13</sup> Williams and Watson (71) have recently reported dissociation of ovalbumin in 50 per cent urea. Burk and Greenberg agreed with Huang and Wu in finding no change in osmotic pressure with this protein, in solutions containing 40 gm. of urea per 100 cc. Their results on zein agree with those of Williams and his collaborators (69).

dilution of this protein brings about its partial dissociation. Interaction with other proteins may produce the same effect (Pedersen (43), and unpublished work). The presence of half size molecules in very dilute solutions may help to explain the departures from normal behavior, reported in studies of the effect of this protein on the activity coefficients of slightly soluble salts (56), and the curious differences between the oxygen combination curves of dilute and concentrated horse hemoglobin solutions (27).

Globin itself has been reported to have half the molecular size of the respiratory protein (51) but other results (72) appear to show that this is only true of urea-dissociated or urea-denatured globins of horse and ox, the hemoglobins of which themselves dissociate in urea solutions. The molecular weight of fetal hemoglobin, known to differ from that of the adult (24, 12), has never been measured.

Confirmation of a marked change in the molecular parameters of hemoglobin when dissolved in 4 M urea has recently been afforded by the dielectric increment measurements on this protein by Oncley and Ferry (personal communication). These investigators also confirm the finding that glycine solutions of far higher dielectric constant than those of urea exert a much smaller effect.

Since the work of Hopkins, it has appeared that many of the effects of urea on proteins are in some manner specific to unsubstituted and partially substituted amides. It can be shown that this specificity is not due to some common *physical* property, such as contribution to the dielectric constant of the solutions. Urea solutions have dielectric constants slightly higher than those of pure water (about 90 at 4 M); the same is probably true of formamide. However, acetamide solutions at concentrations effective in almost totally dissociating hemoglobin have dielectric constants about 10 per cent *lower* than those of water (73). Finally, 2 M glycine (dielectric constant about 124) fails to produce more than a slight dissociation. If part of the potential binding energy between halves of a dissociable protein molecule is electrostatic in origin, a shift of the equilibrium toward dissociation should ensue in solutions of high dielectric constant. Since an electrostatic term in the binding energy (as in the case of a hydrogen ion bound in a weak acid) has not been totally excluded,

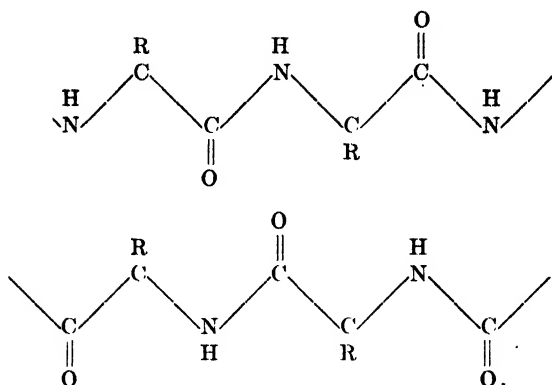
variations in dielectric constant may prove to have greater effects when the protein concentrations are lowered to the range in which considerable dissociation occurs in water alone. Urea solutions, with dielectric constants above those of water, are effective at lower concentrations than the less polar solutions of acetamide. The principal influence of either, however, is clearly not electrostatic and appears to be a property of the amide bond.

The effect of amino acids and certain other molecular dipoles on the solubility of other amino acids, polypeptides, and proteins, has been treated successfully as a function of their effect on the dielectric properties of the solvent (33, 16, 17). Here also the effects of urea are out of all proportion to its dielectric contribution. Its effect on solubility is manifested at much lower concentrations than those at which dissociation becomes considerable, and is as pronounced with pepsin, which does not dissociate, as with hemoglobin, which does.

Mechanisms proposed to account for these phenomena must take into account their specificity to the amide bond. The repeated occurrence of the latter as the peptide link of proteins suggests the significance of this specificity. The x-ray analyses of Astbury have demonstrated that most proteins must be thought of as two- or three-dimensional undulating grids, the woof of which is composed of polypeptide chains (11). The nature of the cross-linkages, constituting the warp of this grid, is still obscure, although interesting hypotheses have been advanced by Astbury, Speakman, Burk, and others. The amide effects suggest that a contribution to the binding energy between parallel chains may arise from strong polarization effects between amide bonds lying opposite one another in regular reversed positions. Large binding forces may originate in this way, since the component polypeptide chains are composed of amino acids with amino groups all in the  $\alpha$  position.

This regular juxtaposition (fragments of two chains are shown below) may give rise to considerable polar association between the chains in much the way that Astbury has suggested may occur within coiled single chains of  $\alpha$ -keratin. It has been estimated that binding forces of as much as 10,000 calories per mole of peptide groups may arise by such polarization (39).





If this type of association between adjacent chains occurs, the effect of amides may depend on their similar association with the elements of the grid at the peptide bonds, *in competition with peptide bonds of adjacent chains*. The association would thus be destroyed or greatly weakened. Removal of the amide by dialysis or dilution would permit reassociation of the component chains. After complete dissociation, however, reassociation may not occur in an orderly way. Chains may come together, not in pairs but in large irregular aggregates. Sufficiently large aggregates would precipitate. The hypothesis which accounts for dissociation may thus also explain the observed behavior of the dialyzed protein. Its application to amide effects on protein solubility will be considered in a later paper.

It has been shown that dissociated hemoglobin possesses all the familiar properties of the normal or native protein. This constitutes strong support for the view which has emerged as the result of other researches in the Upsala laboratory that protein molecular weights may represent aggregates or polymers of smaller units with chemical properties in large measure independent of their state of aggregation.

#### SUMMARY

Native isoelectric horse hemoglobin, in dilute unbuffered salt solutions, is totally dissociated into molecules of half the normal molecular weight when high concentrations of urea or other amides are present in the solution. This conclusion depends on meas-

urements of sedimentation and diffusion constants and partial specific volumes; and is supported by theoretical considerations, and by the failure of higher concentrations to produce a greater apparent effect. The same amide concentrations do not change the molecular weight of another protein, pepsin.

On removal of amides by dialysis, reassociation is observed. Part becomes normal protein but part aggregates into still larger molecules. A fraction of the total protein, the size of which depends on the amide concentration, precipitates irreversibly.

Although certain derivatives of the half weight molecule are no longer stable, spectroscopic tests, and gas capacity and gas affinity measurements show that change in molecular weight is unaccompanied by any evidence of denaturation. When oxidized to methemoglobin, the protein soon changes into an apparent parahematin which possesses the anomalous property of being transformed back to ordinary reduced hemoglobin on reduction with hydrosulfite. The significance of this property for theories of the interrelation of various hemoglobin derivatives is discussed.

Pepsin, like hemoglobin, suffers no changes in specific properties on solution in urea.

The great effects of amides on protein solubility are manifested at much lower concentrations than those at which dissociation occurs; they are as great with pepsin, which does not dissociate, as with hemoglobin. Neither phenomenon is primarily related to the dielectric constants of the media, which are both greater and smaller than those of water. A tentative stereo-polarization mechanism, based on the specificity of amides in producing these effects and on the repeated occurrence of the amide bond in the constituent polypeptide chains of proteins, has been proposed.

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## LIVER LIPIDS OF THE LAYING AND NON-LAYING BIRD\*

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In a previous report (1) it was shown that in the mature female bird actively engaged in egg laying the lipid concentration of the blood is enormously increased above that of the immature female. Values for total lipids as high as 4000 mg. per cent were found in the whole blood of laying birds. Neutral fat (triglycerides) showed the most pronounced rise, although significant changes occurred also in the phospholipid and free cholesterol levels. The rise in the lipid level of the blood is related to ovarian activity, for it first appeared in female birds during puberty and at no time occurred in the male bird from early age to maturity. The degree of ovarian activity, however, does not determine the concentration of lipids in the blood, since in laying birds with increased blood lipid levels no correlation was observed between duration or intensity of egg production and the raised lipid level. In the male and non-laying female substitution of fat for carbohydrate in the diet was without appreciable effect upon all blood lipid constituents except cholesterol esters. In the laying bird the only significant effect of a change of dietary fat from a low to a high level that was observed was a decrease in the variability or degree of fluctuation in the blood levels of neutral fat and free cholesterol.

In the present study the lipid changes in the liver of the bird have been examined. It is shown that in the actively laying fowl lipid changes are not confined to blood alone. An accumulation of fat also takes place in the hepatic tissue.

\* Aided by a grant from the Research Board of the University of California, and by assistance from the Works Progress Administration.

## EXPERIMENTAL

Single comb white Leghorn fowls were used throughout. Their care and treatment, as well as the composition of the high and low fat diets employed, have been described elsewhere (1). The low fat diet contained less than 1 per cent fat, whereas the high fat diet contained approximately 18 per cent fat. In the case of five immature birds, a third diet was used; namely, the stock ration

TABLE I  
*Liver Lipids of Male Birds*

The values are expressed as per cent of fresh tissue.

Bird No.	age	Bird weight	Liver weight	Cholesterol			Total fatty acids	Phospholipids	Residual fatty acids*
				Total	Free	Ester			
Low fat diet									
	days	kg.	gm.						
43	71	0.91	18.5	0.40	0.36	0.04	3.47	2.43	1.81
40	128	1.48	23.1	0.35	0.35	0.00	3.46	3.02	1.44
39	199	1.80	27.0	0.36	0.27	0.09	5.05	1.83	3.76
38	206	1.84	26.9	0.58	0.49	0.09	5.89	2.66	4.04
44	297	2.18	29.0	0.47	0.41	0.06	3.66	2.45	1.97
30	297	2.66	41.9	0.42	0.39	0.03	3.25	2.36	1.65
High fat diet									
87	71	0.95	20.2	0.43	0.35	0.08	2.47	2.43	0.78
70	106	1.22	26.2	0.45	0.38	0.07	3.09		
84	128	1.49	26.1	0.40	0.35	0.05	3.08	3.06	0.99
94	216	2.01	24.9	0.33	0.31	0.03	2.84	2.50	1.14
83	297	2.26	27.7	0.33	0.32	0.01	3.63	1.80	2.42
89	297	2.33	26.0	0.50	0.40	0.10	3.37	2.71	1.48

\* Fatty acids other than those in combination with cholesterol and phospholipids.

of the Poultry Division.<sup>1</sup> This contained approximately 7 per cent fat.

*Determination of Liver Lipids*—Before removal of the liver the birds were killed by breaking their necks. The liver was imme-

<sup>1</sup> This diet was prepared as follows: yellow corn, 52.5 per cent; wheat, 10 per cent; wheat bran, 15 per cent; fish meal, 9.5 per cent; dried milk, 5 per cent; dehydrated alfalfa, 5 per cent; bone meal, 0.5 per cent; oyster shell, 1.5 per cent; salt, 0.5 per cent; sardine oil, 0.5 per cent.

diately cut into small pieces and ground to a fine pulp with a steel spatula. A 10 gm. sample was then placed in a tared vessel containing 20 cc. of 95 per cent ethyl alcohol. For its lipid determination the hepatic tissue was quantitatively transferred to a 125 cc. Erlenmeyer flask. 30 cc. more of ethyl alcohol were then added and the tissue extracted at 55° for 2 hours. The alcohol was decanted through a fat-free filter paper into a 200 cc. glass-

TABLE II

*Liver Lipids of Immature Female Birds*

The values are expressed as per cent of fresh tissue.

Bird No.	Age	Bird weight	Liver weight	Cholesterol			Total fatty acids	Phospholipids	Residual fatty acids
				Total	Free	Ester			
Low fat diet									
	<i>days</i>	<i>kg.</i>	<i>gm.</i>						
01	71	0.73	15.2	0.45	0.39	0.06	4.68	2.40	3.03
42	106	1.08	17.7	0.45	0.43	0.01	3.33	2.45	1.68
12	128	1.32	21.3	0.41	0.36	0.05	3.01	2.39	1.37
High fat diet									
66	71	0.71	16.5	0.47	0.38	0.09	3.45	2.68	1.59
58	106	1.02	20.5	0.45	0.44	0.01	3.64	3.90	1.02
79	128	1.26	16.4	0.43	0.36	0.07	3.49	2.93	1.48
Stock ration									
7015	77	0.70	16.1	0.38	0.38	0.00	3.42	2.72	1.60
7010	77	0.77	15.9	0.46	0.40	0.06	4.07	3.12	1.94
6902	77	0.73	13.7	0.39	0.37	0.03	4.00	2.86	2.06
6929	77	0.92	18.3	0.52	0.50	0.02	3.68	2.82	1.78
6985	77	0.87	17.4	0.44	0.36	0.08	3.76	2.80	1.83

stoppered volumetric flask, and the extraction of the residue was repeated for 1 hour with 50 cc. of alcohol. The second alcohol extract was combined with the first and the tissue residue quantitatively transferred to the filter paper. The residue, after being wrapped in its filter paper, was placed in an all-glass Soxhlet extractor. In this apparatus the liver residue was subjected to continuous extraction with ethyl ether for a period of 12 hours. The Soxhlet extractor was constructed so that the distilling flask



TABLE III  
*Liver Lipids of Laying Birds*

The values are expressed as per cent of fresh tissue.

Bird No.	Age	Matur- ity*	Egg record			Yolks in ovary	Bird weight	Liver weight	Cholesterol			Total fatty acids	Phospho- lipids	Residual fatty acids
			Total eggs laid	Produc- tion rate†	Eggs laid last 7 days				Total	Free	Ester			
Low fat diet														
	days	days					kg.	gm.						
07	199	151	38	79.2	6	5	1.38	30.8	0.40	0.31	0.09	14.44	2.60	7.05
23	217	197	10	50.0	4	6	1.42	27.1	0.41	0.37	0.04	9.35	2.66	1.90
17	219	176	22	51.2	4	6	1.49	27.2	0.52	0.44	0.08	14.70	3.35	12.40
26	219	160	10	66.7†	0	1	1.34	25.3	0.36	0.35	0.01	14.90	2.78	13.03
31	219	206	12	76.9	6	7	1.72	41.3	0.35	0.34	0.01	6.59	2.22	5.10
08	220	201	12	63.2	4	6	1.98	82.0	0.41	0.39	0.01	12.27	2.09	10.86
15	220	173	29	61.7	4	5	1.56	39.5	0.29	0.28	0.01	12.80	2.38	10.21
19	220	190	15	50.0	3	7	1.57	43.2	0.40	0.40	0.00	32.60	1.82	31.35
24	220	160	27	45.0	3	5	1.63	44.2	0.40	0.36	0.04	9.14	2.58	7.37
28	297	167	91	70.0	5	6	1.80	54.1	0.35	0.30	0.06	16.25	2.28	14.67
02	299	160	97	69.8	4	5	1.35	35.7	0.35	0.31	0.04	20.10	2.24	18.57
04	299	160	74	66.7†	5	5	1.68	39.2	0.35	0.31	0.04			
11	299	171	65	54.6†	3	7	1.99	58.1	0.32	0.29	0.04			



held a little over 100 cc. of ether, and the extracting chamber containing the liver residue was refilled every 3 minutes. The ether thus obtained was added to the alcoholic fluid referred to above and the contents of the volumetric flask made to volume with ether at 20°. The mixture was then filtered and an aliquot sample taken for analysis. All solvents employed in this study were purified and freshly distilled before use. The lipid content of this extract was determined by microoxidative procedures. These have been described elsewhere (2).

### Results

*Liver Lipids of Male Bird*—The concentration of the various lipid constituents in the livers of twelve male birds maintained on high and low fat diets is shown in Table I. The level of fat in the diet produced no apparent differences in the amounts of phospholipids or cholesterol in the livers. The major portion of the cholesterol was present in the free form, the esterified portion constituting between 0 and 20 per cent of the total cholesterol.

Although the results recorded are not extensive, they nevertheless suggest that between 70 and 250 days of life age has no influence on the levels of the various lipid constituents in the male bird.

*Liver Lipids of Immature Female Birds*—These results are recorded in Table II. In eleven birds, which were maintained on three different diets containing different amounts of fat, the values obtained for the various lipid constituents were well within the range recorded above for the male bird (Table I).

*Liver Lipids of Mature or Laying Female Bird*—The values obtained for the various lipid constituents of the laying bird are shown in Table III. The effects of high and low fat diets were studied.<sup>2</sup> Ten birds were maintained on the former, thirteen on the latter diet. As in the case of males and immature females, the level of fat in the diet failed to influence the cholesterol or phospholipid content of the liver. A difference, however, did appear in total fatty acids between the birds fed low and those fed

<sup>2</sup> Analyses of the feces showed that the birds on the high fat diet absorbed more than 90 per cent of the fat ingested. The average bird ingested about 80 to 90 gm. of the mash per day and this contained about 15 gm. of fatty acids.

high fat diets. This difference was entirely due to neutral fat. The thirteen birds maintained on the low fat diet contained an average of  $13.37 \pm 2.03$  per cent of liver fatty acids, while the ten birds fed the high fat diet contained an average of  $8.29 \pm 0.85$  per cent fatty acids. Thus a significant difference of  $5.08 \pm 2.20$  per cent fatty acids was observed between the two diets.

Onset of maturity resulted in a pronounced change in the lipid metabolism of the bird's liver. The most striking change was the increase in the level of total fatty acids. Phospholipids and ester cholesterol were unaffected. Although a statistically significant decrease of  $0.050 \pm 0.016$  per cent<sup>3</sup> appeared in the free cholesterol, this small difference is of questionable importance.

#### DISCUSSION

It has already been pointed out that ovarian activity provides a stimulus for mobilization of fat in the *blood* of the domestic fowl (1). The present study shows that the liver of this animal also responds to egg laying by increasing its lipid content. An increase in the fat content of the liver of the laying goose has also been noted by Flock, Bollman, Hester, and Mann (3). The response in lipid activity of the liver of the laying domestic fowl observed in the present study is strikingly significant in view of the fact that in the *immature* bird the ingestion of a diet containing 18 per cent fat failed to raise the lipid content of the liver above that found in similar animals receiving diets with less than 1 per cent fat. Thus, while they readily respond to increased ovarian activity even in the presence of a diet with very little fat, the livers of immature birds contain 3 to 5 per cent fatty acids irrespective of whether the animal ingests a diet containing 18 per cent or less than 1 per cent fat. In the laying bird the mean values for total fatty acid content of the liver were more than double those found in the non-laying birds.<sup>3</sup> The differences were

<sup>3</sup> In several instances, where differences between diet subclasses were not significant, the subclasses could be combined in order to increase the number of observations and thus the precision of the means. Thus the influence of sexual maturity on the cholesterol fractions could be tested without regard to diet, and the mean total fatty acid values for the two groups of laying birds could be compared separately with one mean fatty acid value for all immature females studied.

more than 4 times their standard errors and hence highly significant.

Although both liver and blood respond to laying, the lipid changes in these two tissues are not identical. Neutral fat is principally affected in both tissues. In the blood significant increases also take place in free cholesterol and phospholipids. In the liver the neutral fat is likewise increased, but phospholipids are not affected. Diet apparently influences the degree of lipid infiltration in the liver of the laying bird. Since the lack of appreciable amounts of fat in the diet of the bird does not prevent a lipid infiltration in blood and liver, it would seem that the increased amounts of lipids from non-fat precursors can be a major metabolic process in the laying bird.

*Relation of Ovarian Activity to Lipid Infiltration in Liver*—Three measures of ovarian activity were employed: (1) the number of actively growing yolks found at autopsy; (2) the number of eggs laid during the 7 days preceding removal of the liver; (3) rate of production; i.e., the total number of eggs laid per 100 days from the onset of maturity. Pause periods, which were defined according to Lerner and Taylor (4) as intervals of 7 or more successive days during which no eggs were laid, were not included in the total number of days of the mature period.

Correlation coefficients were calculated between liver lipid levels of laying birds and the three measures just named. The amounts of liver cholesterol or phospholipids were probably not correlated with any of the measures of ovarian activity studied, although isolated statistically significant correlation coefficients were obtained. For example, a significant correlation of  $-0.665$  was found between free cholesterol and the number of yolks found in the ovary of the birds fed the low fat diet, but the equivalent correlation coefficient for birds fed the high fat diet had the very small value of  $+0.079$ , and coefficients with the other two measures were likewise not significant. For the birds on the high fat diet, however, significant positive correlation coefficients were obtained between total fatty acids and the number of eggs laid during the last 7 days ( $r = +0.753$ ), and between total fatty acids and rate of production ( $r = +0.643$ ). It is interesting to note that, if the rate of production had not been corrected for pause periods, the above correlation would have been obliterated,

an observation that lends further support to the conclusion of Lerner and Taylor (4) that pause and rate are entirely separate characters, and that pause periods should be excluded from any method of measuring rate of egg production.

In birds maintained on the low fat diet no significant correlations were obtained between fatty acids and any of the three measures of ovarian activity, although the trends were in the same direction as in the case of the high fat diets. The total variability of fatty acids in the low fat diet was greater than in the high fat diets; this excess variability apparently masked the correlations. This observation may be compared with the results on blood lipids (1), where an interaction between dietary fat, ovarian activity, and blood fatty acid variability was found.

#### SUMMARY

1. A study was made of the relation of diet, sex, and ovarian activity to the liver lipids of the single comb white Leghorn fowl.

2. The same concentration of liver lipids, namely free and esterified cholesterol, phospholipids, and neutral fat (triglycerides), was found in the mature and immature male bird and in the immature female bird.

3. The onset of maturity led to a pronounced increase in neutral fat in the liver of the female bird. No change was observed in the phospholipid or in the cholesterol ester content of the liver.

4. An increase in the fat content of the diet led to a decrease in the neutral fat content of the liver of the bird actively engaged in egg laying. The neutral fat content was more variable in the liver of the birds fed a low fat diet than in those fed a high fat diet.

5. The significance of correlation coefficients obtained between the various liver lipid constituents and measures of *ovarian activity* is discussed.

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## **RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM \***

### **II. THE RÔLE OF THE STOMACH, SMALL INTESTINE, AND LARGE INTESTINE IN PHOSPHOLIPID METABOLISM IN THE PRESENCE AND ABSENCE OF INGESTED FAT**

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It has been shown (1-3) that radioactive phosphorus provides a method for directly comparing the phospholipid turnover occurring in various tissues. In a previous communication from this laboratory the content of "labeled phospholipid" in liver, kidney, gastrointestinal tract, brain, and the whole animal was shown at several intervals after the administration of sodium phosphate that had been marked by the inclusion of radioactive phosphorus (3). Phospholipid formation was not confined to animals ingesting fat; a rapid turnover of this lipid constituent also occurred in all tissues examined in rats previously fasted for as long as 40 hours. Three tissues, namely the gastrointestinal tract, liver, and kidney, were particularly active in phospholipid turnover, and the shapes of the curves representing the phospholipid content as a function of time were similar for these three organs, showing a maximum content between 6 and 12 hours after the ingestion of the labeled phosphate. From the curves showing phospholipid content as a function of time, it would seem that the tissues of the animal body might be divided into two classes: (1) those with a high activity per gm. of tissue, and (2) those with a low activity. The curves for the former were characterized by a sharp rise and decline, while the latter showed a slower rise that continued for many

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hours after the administration of the labeled phosphorus. Although the entire gastrointestinal tract was used in the previous investigation, it was recognized that this consists of several parts differing structurally as well as functionally. In view of the two distinct types of phospholipid activity found, it was deemed worth while to examine in detail the rates of phospholipid turnover by various parts of the gastrointestinal tract; namely, stomach, small intestine, and large intestine. It is shown in this investigation that, *in the absence* as well as in the presence of fat, only one part, namely the small intestine, possesses a high phospholipid activity, the stomach and large intestine accounting for very little of the turnover found in the entire gastrointestinal tract. Thus, even in the absence of ingested fat, a sharp difference in phospholipid metabolism of the gastrointestinal tract is established for various parts.

#### EXPERIMENTAL

The radioactive phosphorus ( $P^{32}$ ) used throughout this study was prepared by bombardment of phosphorus with deuterons accelerated in the cyclotron of Professor E. O. Lawrence. It was administered as a solution of pure  $Na_2HPO_4$ .

The lipids were extracted from the tissues and the phospholipids precipitated in the manner previously described (3). The mounting of the precipitated phospholipid has also been noted elsewhere (3). Its content of radioactive phosphorus was determined by means of a small, thin-walled, aluminum Geiger counter. The sensitivity of the counter was checked before and after each measurement by means of a standard thorium source (3).

*Fat-Fed Rats*—Twenty-two rats that had been fasted for 40 hours received 1 cc. of olive oil by stomach tube. Phosphorus was administered by two paths, subcutaneously and by stomach tube. The administration of 2 cc. of  $Na_2HPO_4$  containing 6 mg. of P and  $1.01 \times 10^5$  radioactive units<sup>1</sup> was always made within a few minutes after the oil. The labeled phospholipid content found at various intervals in the stomach, small intestine, and large intestine is shown in Figs 1 and 2. Each point on the curve represents the average of the results obtained from two rats.

<sup>1</sup> 1 radioactive unit =  $2 \times 10^{-12}$  curie.

While more data are necessary to furnish unique curves, the general trend is nevertheless unmistakable. In the early hours the labeled phospholipid content of the small intestine was somewhat smaller in the animals that received phosphorus subcutaneously than in those that received it by stomach tube. When compared in respect to weight of tissue, the labeled phospholipid content of the stomach and large bowel is but a small fraction of that found in the small intestine. It would seem that the phospholipid turnover ascribed to the gastrointestinal tract is a func-

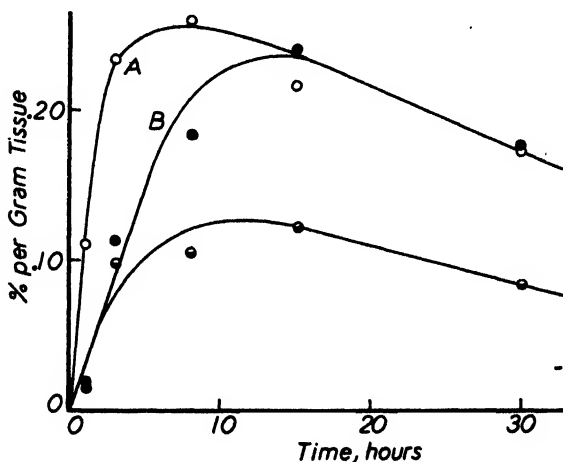


FIG. 1. Labeled phospholipid content of the small intestine. The ordinates represent the per cent of administered phosphorus found as phospholipid per gm. of tissue. ○, obtained from rats fed both phosphorus and olive oil by stomach tube; ●, from rats that received olive oil by stomach tube and phosphorus subcutaneously; ◐, from rats that received phosphorus subcutaneously.

tion of the small intestine. So it is interesting to note that the shape of the curve obtained for the small intestine resembles those noted previously for liver and kidney, tissues very active in phospholipid formation. The stomach and large intestine showed a continued rise within the time limit of the experiment; namely, 30 hours. A similar rise was previously noted for the carcass (3), a term used to include tissues other than liver, kidney, and gastrointestinal tract.

*Endogenous Phospholipid Metabolism in Various Parts of the*

*Gastrointestinal Tract*—In view of the fact that the absorption of fat takes place in the small intestine, the question arises whether the active synthesis of this portion of the tract as compared with the rest of the intestine is a result of the fat which was administered simultaneously with the radiophosphorus. That this is not the case is shown by the following experiment. In several experiments groups of ten rats were fasted for 40 hours and then

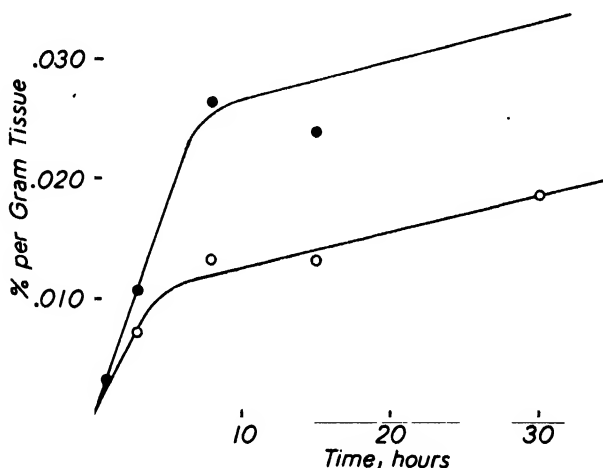


FIG. 2. Labeled phospholipid content of stomach and large intestine. The ordinates represent the per cent of administered phosphorus found as phospholipid per gm. of tissue. O, values for stomach of rats that received phosphorus subcutaneously; ●, for large intestine of rats that received phosphorus subcutaneously. Curves similar to the above in shape and in order of magnitude were obtained for stomach and small intestine in rats fed olive oil along with phosphorus (administered either subcutaneously or *per os*).

injected subcutaneously with 2 cc. of  $\text{Na}_2\text{HPO}_4$  solution containing 8 mg. of P and  $1.57 \times 10^5$  radioactive units. The phospholipid formed in various parts of the gastrointestinal tract for one such group is shown in Figs. 1 and 2. The results demonstrate clearly that even in the absence of ingested fat the small intestine is still the most active part of the gastrointestinal tract with respect to phospholipid turnover, the amount formed in these circumstances being approximately 30 to 40 per cent of that

formed when fat is fed. While the term "endogenous" is used here to describe phospholipid formation when no fat is fed, it should be noted that the significance of traces of fat secreted into the lumen has not been ruled out. It has been shown that lipids are secreted into the small intestine (4) and that the chief source of the fat in the thoracic lymph of the fasted animal is the bowel (5).

*Effect of Excision of Gastrointestinal Tract upon Formation of Phospholipid by Liver*—In view of the intimate circulatory con-

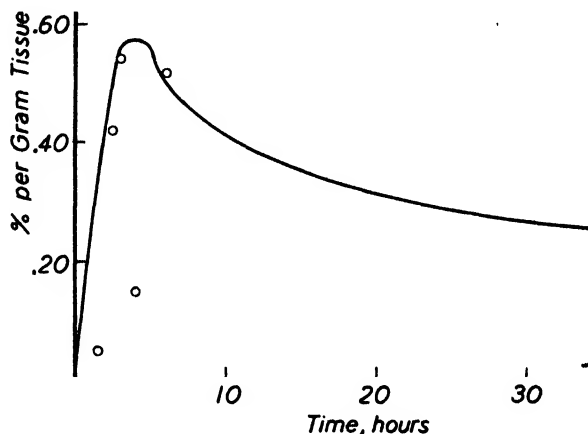


FIG. 3. Labeled phospholipid content of the liver of rats in which both kidneys and entire gastrointestinal tract had been removed. The curve represents the labeled phospholipid content per gm. of tissue obtained in normal rats fed phosphorus by stomach tube (3); O, obtained from rats that received phosphorus subcutaneously after both kidneys and the entire gastrointestinal tract had been removed. The points were obtained from single animals except the one shown at 6 hours, which represents the average of two animals.

nection between liver and intestine—the venous outflow from the latter being received by the liver—it seemed by no means unlikely that the rapid turnover occurring in the intestine might have some connection with the phospholipid found in the liver. Although it has been suspected for some time that the liver is capable of synthesizing phospholipid (6), direct evidence in support of this claim has thus far been difficult to obtain, since the methods employed for following phospholipid formation necessitated the

administration of a foreign fatty acid by way of the intestinal tract, a tissue that, as already noted, forms phospholipid rapidly even in the absence of fat feeding. The fact that *parenterally* administered phosphorus is so readily incorporated into the phospholipid molecule made feasible the study of the formation of labeled phospholipid in the liver of animals deprived of both kidneys and gastrointestinal tract, two of the three tissues that are very active in the deposition of phospholipid in response to administered phosphorus.

In a number of rats both kidneys and the entire gastrointestinal tract were excised. Between 2 and 5 hours after the operation six such animals received subcutaneously 1 cc. of a solution containing 4 mg. of phosphorus in which were present  $5.36 \times 10^4$  radioactive units. The livers were removed from 1.5 to 6 hours after the injection of the  $\text{Na}_2\text{HPO}_4$ . The labeled phospholipid formed in the livers of these rats is shown in Fig. 3. The curve shows the phospholipid content of the liver of the intact rat fed phosphorus by stomach tube (previously reported (3)), whereas the circles represent the values obtained in the present study with animals in which the kidneys and gastrointestinal tract were excised. While the close correspondence between the circles and the curve is probably coincidental, the results suggest that the endogenous phospholipid metabolism in the liver is not markedly influenced by the process occurring simultaneously in the small intestine.

#### SUMMARY

1. With radioactive phosphorus as indicator, a comparison was made of the phospholipid turnover by various parts of the gastrointestinal tract.

2. In the *absence* as well as the presence of ingested fat, the major part of the phospholipid turnover by the gastrointestinal tract can be ascribed to the small intestine, the stomach and large intestine playing but minor rôles in this type of metabolism.

3. The removal of tissues very active in phospholipid turnover, namely the gastrointestinal tract and kidneys, does not markedly influence the phospholipid turnover by the liver.

The preparation of the radioactive phosphorus samples used in this study was made possible by a grant to Professor Ernest O.

Lawrence and coworkers of the Radiation Laboratory by Mr. Max Schott.

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# A STUDY OF THE PERIODIC ACID OXIDATION OF STARCHES AND DEXTRINS AS A MEANS OF DETERMINING MOLECULAR SIZE\*

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Jackson and Hudson (1) have shown that corn-starch is oxidized rapidly with periodic acid, that the reaction is practically quantitative, and that the cleavage of the glucose molecules in the starch chain is the same as in the simpler glycosidic derivatives (2-4). The reaction has been under investigation in this laboratory as a means of determining the size of the starch molecule and the nature of the terminal glucose groups. The oxidations were made on gelatinized starches and on fractionated dextrans, as contrasted to the investigations of Jackson and Hudson which were made on raw, granular starch.

The most generally accepted conception of starch pictures it as consisting of a chain of glucopyranose units joined in  $\alpha$ -1,4 linkages (5). This configuration requires two terminal glucose units which differ from the rest in that one would have a free aldehyde group and the other an additional free hydroxyl group. While there is some question as to the configuration of the terminal units, advantage has been taken of this differentiation for determining the chain length of the starch molecule.

Haworth and his collaborators (6), as well as others (7), have determined the proportion of non-reducing end-units in fully methylated starch and have concluded that the number of glucose units in a starch chain is approximately twenty-five to thirty. Hess and Neumann (8), working with cellulose, have questioned

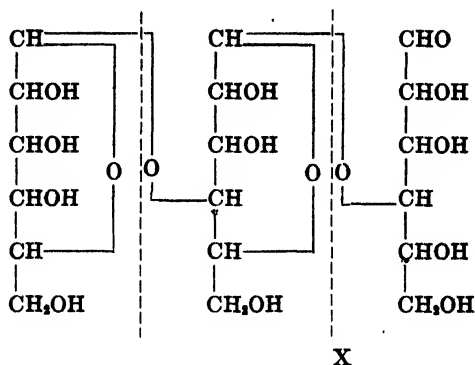
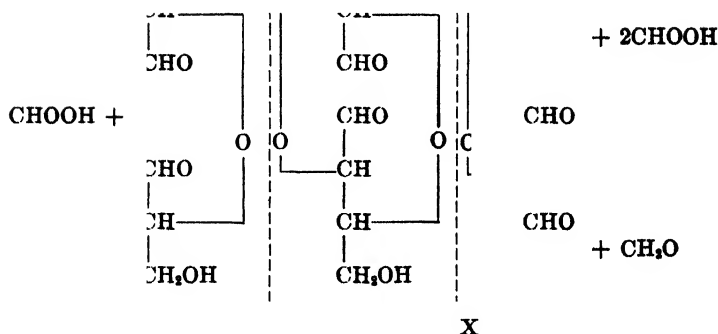
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the significance of Haworth's end-group method, particularly in connection with the pretreatment of the polysaccharide before methylation and with the distillation method of fractionating the glucose ethers.

Farrow and his collaborators (9), working with the opposite end of the molecule, have demonstrated that starch has a slight reducing value. From the ratio of the reducing power of the sample and that of a substance of known chain length, such as maltose, they have arrived at a value of 460 to 1470 glucose units for the chain length. The wide discrepancy between the values obtained by the two groups of workers has been explained by assuming that in starch many of the chain molecules are held together in parallel by coordinate linkages and thus aldehyde groups which are chemically free may be protected and their functioning in the usual manner prevented (10). This is essentially the hypothesis of aggregation proposed by Haworth and his coworkers (11) to account for the disagreement of their value for the molecular size of starch with the much higher ones determined by the physical methods of Svedberg (12) and of Staudinger (13). Farrow *et al.* (9), however, discredit the theory of aggregation on the basis that in the early stages of starch hydrolysis a slight increase in reducing power gives rise to a large decrease in average chain length. Consequently there is a rapid initial diminution in viscosity, a phenomenon which hitherto has been attributed to the formation of "soluble starch" by a process of disaggregation. Furthermore, Farrow *et al.* have shown that the rate of starch hydrolysis, with one exception, is a linear function of time.

When the periodic acid oxidation is applied to starch, it is apparent that, while the intermediate glucose units in the chain will be oxidized as reported by Jackson and Hudson (1), the terminal units will give rise to 3 molecules of formic acid (2, 14) and 1 of formaldehyde (14), as shown in the accompanying formulas. It is also apparent that the quantity of formaldehyde formed should correlate with the reducing power of the starch or dextrin molecule. No attempt was made to measure the formic acid produced by the oxidation, since it was difficult to recover and there was available no good method for the estimation of the small quantities recovered.

*Oxidation of Theoretical Starch Molecule by Periodic Acid*(HIO<sub>4</sub>)

The oxidized starch obtained by the action of periodic acid was very similar to that prepared by Jackson and Hudson, although in this case gelatinized starch was used rather than raw, granular starch. Derivatives of glyoxal were prepared from the hydrolysis products of the oxidized starch, which tended to substantiate the conclusion that the rupture of the non-terminal glucose units of starch by periodic acid takes place between carbon atoms (2) and (3). Conclusive proof for this, however, depends on the identification of *d*-erythrose as a hydrolysis product of the oxidized starch. The *d*-erythrose has not been found, possibly because of

its instability toward the hydrolyzing agent or because of its difficulty of isolation in the presence of glyoxal and other degradation products.<sup>1</sup>

#### EXPERIMENTAL

*Preparation of Dextrin Samples*—The dextrin samples were prepared from a series of six commercial dextrans. These dextrans were designated by the letters A, B, C, D, E, and F, the order of increasing conversion being from A to F.

The dextrans were fractionated according to their solubilities in water and alcohol-water mixtures. Since the solubilities of the dextrans varied greatly, the number of fractions made was not always the same. With Dextrans A and B, only the fractions soluble and insoluble in cold water were made. Dextrans C and D were divided into a cold water-insoluble fraction, one insoluble in 52 per cent alcohol, and one soluble in 52 per cent alcohol. Dextrans E and F were separated into portions which were cold water-insoluble, insoluble in 52 per cent alcohol, insoluble in 75 per cent alcohol, and soluble in 75 per cent alcohol.

The fractionations were made according to the following procedure: 60 gm. samples of the dextrin were shaken for 2 hours with 600 cc. of distilled water. The insoluble portions were then filtered off with suction and washed with a very small portion of water. The 52 per cent alcohol-insoluble fractions were prepared by pouring the cold water extracts into a volume of 95 per cent alcohol 1.5 times as large. The precipitate formed was separated first by settling and finally by centrifuging when the solid would not separate out completely. The solutions were then poured into an additional amount of 95 per cent alcohol to make the total volume of alcohol 5 times as large as the volume of the original cold water extract. The precipitates were separated in the same manner as was described for the 52 per cent alcohol

<sup>1</sup> Dr. E. L. Jackson and Dr. C. S. Hudson inform us by private communication that they have identified glyoxal and *d*-erythrose, the former as its phenylosazone melting at 170–171° and its benzylphenylosazone melting at 199–200°, both obtained in yields of 25 to 30 per cent; the erythrose, after oxidation with bromine water, was identified as brucine *d*-erythrinate (yield 20 to 25 per cent; m.p. 210–211°;  $[\alpha]_D^{20} = -22.6^\circ$  in water) from which *d*-erythronic lactone (m.p. 104–105°;  $[\alpha]_D^{20} = -73.3^\circ$  in water) was prepared. Their publication will appear shortly.

TABLE I

*Relation of Amounts of Oxygen Used to Total Equivalents of Palmitic and Phosphoric Acids in Various Starch and Dextrin Samples*

The equivalents of palmitic and phosphoric acids were obtained by dividing the percentages of fat and phosphoric acid in the samples by the molecular weights of the palmitic and phosphoric acid radicals respectively. The theoretical weight of oxygen is 0.0107 gm.

Sample	Equivalents of palmitic acid	Equivalents of phosphoric acid	Total equivalents of acid	Weight of oxygen used gm.
Corn-starch.....	0.0025*	0.0006†	0.0031	0.00642
Rice starch.....	0.0032*	0.0002†	0.0034	0.00583
Wheat ".....		0.0015†		0.00552
Potato ".....	0.0002*	0.0025†	0.0027	0.00486
Dextrin B, fraction soluble in H <sub>2</sub> O.....	0.0001‡	0.0010§	0.0011	0.00778
Dextrin B, fraction insoluble in H <sub>2</sub> O.....	0.0025‡	0.0006§	0.0031	0.00627
Dextrin C, fraction insoluble in H <sub>2</sub> O.....	0.0027‡	0.0006§	0.0033	0.00678
Dextrin C, fraction soluble in H <sub>2</sub> O, insoluble in 52% alcohol...	0.0001‡	0.0002§	0.0003	0.00736
Dextrin C, fraction soluble in 52% alcohol.....	0.0001‡	0.0010§	0.0011	0.00916
Dextrin E, fraction insoluble in H <sub>2</sub> O.....	0.0037‡	0.0009§	0.0046	0.00675
Dextrin E, fraction soluble in 52% alcohol, insoluble in 75% alcohol.....	0.0000‡	0.0002§	0.0002	0.00746
Dextrin F, fraction insoluble in H <sub>2</sub> O.....	0.0075‡	0.0012§	0.0087	0.00728
Dextrin F, fraction soluble in 52% alcohol, insoluble in 75% alcohol.....	0.0000‡	0.0002§	0.0002	0.00750

\* Calculated from the value of the fat content given by Taylor and Nelson (16).

† Calculated from the value of the P<sub>2</sub>O<sub>5</sub> content given by Samec and Haerdtl (17).

‡ Calculated from the value of the fat content determined by the method of Taylor and Nelson.

§ Calculated from the value of the phosphorus content determined by the method of Truog and Meyer (18).

fractions. The solutions were then evaporated to dryness under reduced pressure, in order to obtain the soluble fractions. The fractions were all dried at about  $50^{\circ}$  and then used for the analyses. Moisture determinations were made on the fractions, so that all of the results are calculated on the dry basis. The fractions precipitated from alcohol usually separated out as heavy syrups which, on drying, became white, brittle solids.

*Determination of Periodic Acid Required to Oxidize Starch and Dextrin Samples*—The periodic acid solutions used were prepared by dissolving sodium paraperiodate in 1.5 times the calculated quantity of sulfuric acid and were standardized by the method of Fleury and Lange (15). Accurate standard solutions could be

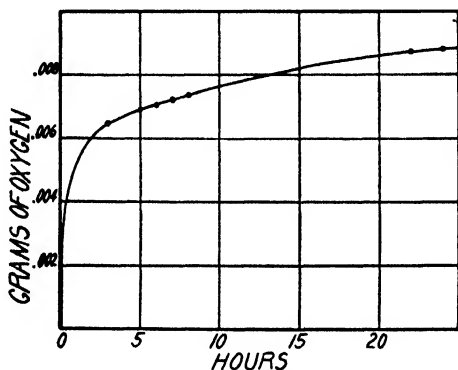


Fig. 1. Rate of oxidation of the water-insoluble fraction of Dextrin B

made by merely weighing out the sodium paraperiodate after it had once been standardized. The oxidation was carried out with corn, rice, wheat, and potato starches as well as with a number of the dextrin fractions. 25 cc. portions of the boiled starch or dextrin solutions containing 0.1 gm. of material were treated with 25 cc. portions of a 0.0636 M solution of periodic acid at  $25^{\circ}$  for definite intervals of time. The periodic acid remaining at any time was determined by the methods of iodometry (15). The amounts of periodic acid used, expressed as equivalent gm. of oxygen, are shown in Table I. The values given were determined over a period of 7 hours; during this time the consumption of periodic acid increased rapidly and after this period it increased very slowly but continuously (Fig. 1).

The quantity of oxygen necessary for the oxidation can be calculated; while the terminal glucose units increase this value, the increase is practically negligible for chain lengths of more than ten units. Such calculations, however, ignore the presence of phosphoric and fatty acid ester linkages in the starch which might mask some of the glycolic groupings and hence block the action of the periodic acid. Evidence for this is seen if the sum of the equivalents of palmitic and phosphoric acids is compared with the equivalents of oxygen used for the several samples (Table I). As a rule the insoluble samples which are high in fat and phosphorus require less oxygen than the more soluble ones containing smaller amounts of fat and phosphorus. The amounts of oxygen used by the latter samples were in fair agreement with the calculated value.

*Preparation and Properties of Oxidized Starch*—1 liter of a 1 per cent boiled corn-starch paste was mixed with 100 cc. of a 0.563 M periodic acid solution and allowed to stand for 2 days at 25°, during which time a jelly-like precipitate coagulated in the bottom of the vessel. After the supernatant cloudy liquid was siphoned off, the residue was triturated with distilled water and then filtered by suction. This process was repeated three to four times to insure removal of the inorganic salts and acids. On drying at about 50°, the oxidized starch became a hard, brittle, white solid which amounted to 65 per cent of the original starch. It was insoluble in cold water but dissolved gradually in boiling water with the exception of a slight amount of dark, flaky material which contained some extractable fat. On evaporation of the water solution a colorless, transparent film resulted. A 0.1 gm. sample was equivalent to 0.091 gm. of glucose in reducing power. It was rapidly resinified by alkalis. On being heated with dilute acids, the material swelled and then dissolved slowly, producing a solution with a slight yellow color which became darker as hydrolysis progressed.

*Identification of the Glyoxal*—1 gm. of the oxidized starch was refluxed for 3 hours with 25 cc. of 0.004 N sulfuric acid. The acid was removed with barium carbonate and the filtrate treated with 4 gm. of phenylhydrazine hydrochloride and 6 gm. of sodium acetate. A voluminous orange-yellow precipitate formed at once. The color as well as the solubility in hot benzene of the phenyl-

hydrazine precipitate varies with the degree of hydrolysis of the oxidized starch which will itself give a light yellow phenylhydrazine precipitate insoluble in hot benzene.

The orange-yellow precipitate on treatment with a small volume of alcohol yielded a residue of light yellow color which, on being filtered and twice recrystallized from hot benzene, gave a precipitate of light yellow, elongated, hexagonal plates, in some cases gathered into spherical clusters, m.p. 172–175°. This product was found to be glyoxal osazone.

*Analysis—*

$C_{14}H_{14}N_4$ . Calculated, N 23.25; found, N 23.00, 23.05

A second precipitate obtained by using benzylphenylhydrazine was fractionated by solution in hot benzene and addition of petroleum ether till the solution became turbid. The fraction obtained on cooling was recrystallized in the same way and yielded a precipitate of fine white needles, m. p. 195° (19). It was found to be glyoxal benzylphenylosazone.

*Analysis—*

$C_{28}H_{28}N_4$ . Calculated, N 14.20; found, N 13.91, 13.97

*Analysis for Formaldehyde—*The amounts of formaldehyde produced by periodic acid oxidation from a number of the dextrin fractions were measured to ascertain whether there was any agreement between the experimental values and those calculated from the average chain lengths determined by Farrow's method (9).

A 0.2 to 0.8 gm. sample of the dextrin fraction to be oxidized after boiling in 30 cc. of water and cooling was treated with 20 cc. of a 0.145 M periodic acid solution. The mixture was allowed to stand at 25° for exactly 24 hours.

The formaldehyde was removed from the reaction mixture by steam distillation under diminished pressure as described by Criegee (20). Each distillation was carried out in exactly the same manner, with the temperature of the steam generator at 60–65°, the temperature of the reaction flask at 35–40°, the receiving vessel cooled by ice, and the pressure adjusted at about 6.5 cm. of mercury. Under these conditions, 65 cc. of distillate collected in about 1 hour. A series of distillations was carried out

as described above with 50 cc. portions of a standard formaldehyde solution having the same acidity as the actual samples, and it was found that 37.7 per cent of the formaldehyde was carried over in 65 cc. of distillate with a variation of about 1 per cent.

TABLE II

*Correlation of Quantities of Formaldehyde Produced by Periodic Acid Oxidation of Various Dextrin Fractions with Chain Lengths As Determined by Farrow's Copper Reduction Method*

Dextrin sample	Reducing values*	Average chain length†	Amounts of formaldehyde	
			Calculated from chain lengths‡	Determined experimentally§
			mg.	mg.
B, fraction insoluble in H <sub>2</sub> O . . . . .	30.65	134.0	0.53	0.47
C, " " " " " " . . . . .	42.70	96.2	0.74	0.94
D, " " " " " " . . . . .	71.25	57.75	1.24	1.59
" " soluble in H <sub>2</sub> O, insoluble in 52% alcohol . . . . .	55.70	73.60	0.97	0.79
E, fraction insoluble in H <sub>2</sub> O . . . . .	108.90	37.80	1.89	1.64
" " soluble in H <sub>2</sub> O, insoluble in 52% alcohol . . . . .	75.33	54.50	1.31	1.41
E, fraction soluble in 52% alcohol, insoluble in 75% alcohol . . . . .	124.63	33.00	2.16	2.10
F, fraction insoluble in H <sub>2</sub> O . . . . .	150.20	27.30	2.64	2.85
" " soluble in 52% alcohol, insoluble in 75% alcohol . . . . .	160.13	25.60	2.81	2.39

\* Expressed as  $RCu$  values.  $RCu$  is defined as the number of mg. of copper reduced by 1 gm. of starch. For modified starches and sugars the unit of weight is taken as the weight equivalent to 1 gm. of starch.

† Calculated from  $RCu$  values. The  $RCu$  of maltose is 2055. Hence to find the average chain length of the molecules of a sample whose  $RCu$  is 100, for example, use the equation  $100X = 2 \times 2055$ .

‡ Theoretical amounts calculated from chain lengths on the basis of a 0.3 gm. sample.

§ Determined on the basis of a 0.3 gm. sample.

The formaldehyde in the distillate was estimated by the method of Vorlander (21) with dihydrodimethylresorcinol (dimedon). The value obtained was divided by 0.377 to correct for the inefficiency of the distillation. The results are shown in Table II.



## DISCUSSION

The agreement between the experimentally determined amounts of formaldehyde produced by periodic acid oxidation and the amounts calculated from the average chain lengths as determined by Farrow's method (Table II) indicates that these two methods must be reliable for the determination of the reducing terminal glucose units.

The suitability of these methods for the estimation of the average chain length of starch or dextrin molecules depends on the assumption that each molecule terminates in a reducing glucose residue. The validity of this assumption is strongly upheld by the arguments of Farrow as to chain length and is further supported by the following considerations.

The most highly modified form of starch sample oxidized was the fraction of Dextrin F, soluble in 52 per cent alcohol and insoluble in 75 per cent alcohol. It has a copper-reducing value ( $R_{Cu}$ ) of 160.3, gives a red color with iodine, and on the basis of the formaldehyde produced by periodic acid oxidation or on the basis of its reducing value has an average chain length of about twenty-five glucose units. The properties of this dextrin fraction, especially those of solubility and reducing power, make it highly improbable that its molecules exist in a state of aggregation. In addition it would seem probable, from the fact that in starch hydrolysis the reducing power has been shown to be a linear function of time, that each of the molecules of this dextrin terminates in a reducing glucose residue with a free unprotected aldehyde group. If this is the case, then the value of twenty-five glucose units given for the average chain length must be reliable. The same argument can be advanced for any one of the dextrin samples which were studied and it is seen that the mean chain lengths run as high as 134 glucose units, which is several times the value of twenty-five obtained by Haworth for starch itself.

Since the estimation of chain length by Farrow's method appears to be reliable with the dextrin fractions, it seems logical to conclude that it is equally reliable with starch, or at least that the starch molecule has an average chain length considerably greater than that of any of the dextrin fractions studied. This would explain why it was impossible by the method used to detect any formaldehyde produced from unmodified corn-starch by

periodic acid oxidation. If corn-starch molecules have an average chain length of 1150 glucose units as determined by Farrow's method, it would require 6 gm. of starch to produce 1 mg. of formaldehyde by periodic acid oxidation. Experiments on such a scale would be inexpedient, owing to the difficulty of quantitatively estimating the formaldehyde present in the large volume of reaction mixture.

#### SUMMARY

The periodic acid consumed in the oxidation of several starches and a series of dextrin fractions was found to vary inversely with the total quantity of fat and phosphorus in the samples. The rate of oxidation of all the samples was very rapid during the first 3 hours but became slight by the 7th hour when the amounts of oxygen consumed by the samples with low fat and phosphorus content approached the theoretical value.

Derivatives of glyoxal were prepared from the hydrolysis products of oxidized starch, which indicates that periodic acid cleaves the non-terminal glucose units of starch between carbon atoms (2) and (3) as pictured by Jackson and Hudson.

Correlation between the amounts of formaldehyde produced by periodic acid oxidation and the reducing values of nine dextrans was demonstrated, indicating that the two measurements are reliable for the determination of reducing terminal glucose units. The reliability of the two measurements for estimating average chain length of dextrans and starches is discussed, with the conclusion that starches must have average chain lengths considerably greater than twenty-five units, as proposed by Haworth.

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## SYNTHESIS OF 5-PHOSPHO-*d*-ARABINOSE

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(Received for publication, January 24, 1938)

In the formulæ below (I to V) are presented the intermediate steps involved in the biological oxidation of glucose as postulated by Warburg *et al.*,<sup>1</sup> Lipmann,<sup>2</sup> and Dickens.<sup>3</sup>

This theory then includes an assumption that 5-phospho-*d*-arabinose undergoes an oxidation analogous to that of 6-phosphoglucose. In order to make it possible to test this assumption experimentally 5-phospho-*d*-arabinose has now been prepared synthetically. The intermediate steps in the synthesis of this substance are given in the accompanying formulæ (VI to XIII).

The substance was isolated in the form of an analytically pure, amorphous barium salt which was transformed into a crystalline brucine salt. It is hoped that, with larger quantities of brucine salt available, the barium salt will be obtained therefrom in crystalline form.

### EXPERIMENTAL

*Preparation of Monoacetone d-Arabinofuranose*—This compound was prepared from *d*-arabinose according to the directions of Levene and Compton<sup>4</sup> for the synthesis of monoacetone *l*-arabinofuranose.

*5-Phospho-d-Arabinose*—15 cc. of dry pyridine, cooled to just above the freezing point, were quickly added to a solution of 2.5 gm. of phosphorus oxychloride contained in a large test-tube

<sup>1</sup> Warburg, O., Christian, W., and Griese, A., *Biochem. Z.*, **282**, 157 (1935).

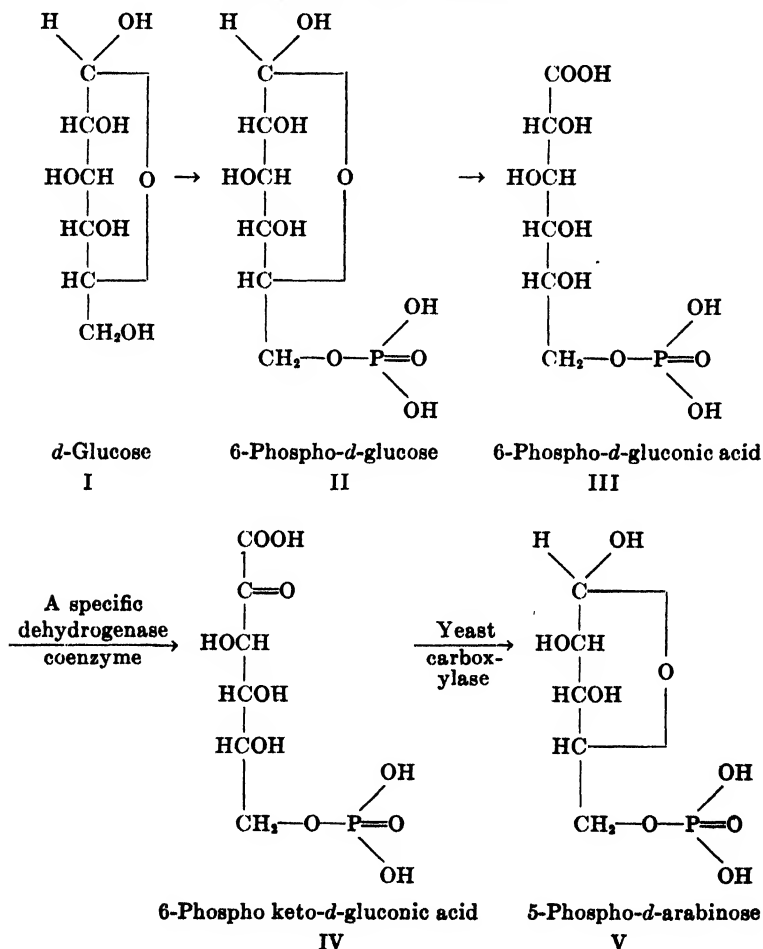
<sup>2</sup> Lipmann, F., *Nature*, **133**, 588 (1936).

<sup>3</sup> Dickens, F., *Nature*, **133**, 1057 (1936).

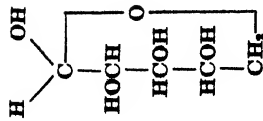
<sup>4</sup> Levene, P. A., and Compton, J., *J. Biol. Chem.*, **116**, 189 (1936).

and cooled to  $-30^{\circ}$  to  $-40^{\circ}$ . The solid was dissolved by removing the tube from the cold mixture and warming with the hand and then recooled to  $-30^{\circ}$  to  $-40^{\circ}$ . To 80 cc. of dry pyridine,

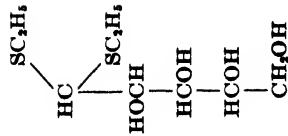
*Biological Oxidation of Glucose*



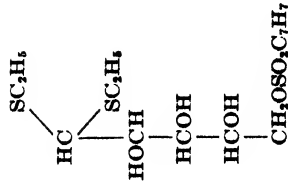
4.8 gm. of monoacetone *d*-arabofuranose were added and this solution was cooled to  $-40^{\circ}$ . The two solutions were quickly mixed together by pouring the sugar solution into the oxychloride solution and then kept in an ice and salt bath for 2 hours.



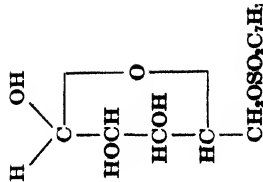
VI. *d*-Arabinose



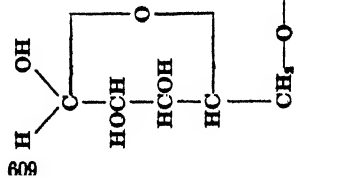
VII. *d*-Arabinose diethyl mercaptal



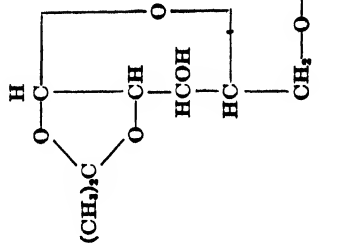
VIII. 5-Tosyl arabinose mercaptal



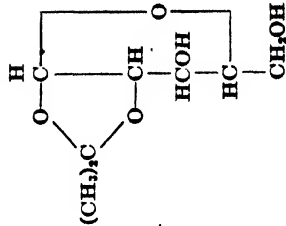
IX. 5-Tosyl arabinose



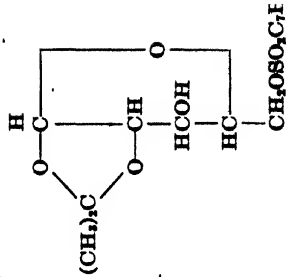
XIII. 5-Phospho-*d*-arabinose



XII. 5-Phospho acetone arabinose



XI. 1,2-Monosaccone arabofuranose



X. 5-Tosyl acetone arabinose

20 cc. of 90 per cent aqueous pyridine were added dropwise to this mixture, the temperature being kept below  $-20^{\circ}$ . Ice and water were added and the solution made alkaline with saturated barium hydroxide solution. The pyridine was removed by concentrating the solution under diminished pressure with occasional addition of water.

The acetone group was removed by making the solution 0.3 N acid with sulfuric acid and then heating at  $90^{\circ}$  until the free sugar content, as determined by the Willstätter method, became constant. This required approximately 2 hours. The solution was now cooled and the halogen removed by treating with 6 gm. of silver carbonate, filtering, and treating with hydrogen sulfide to remove the silver ion.

After removal of the excess hydrogen sulfide by aeration the solution was made alkaline to thymolphthalein with saturated barium hydroxide. The barium sulfate was separated by centrifuging, and the solution was concentrated to about 150 cc. and filtered. The filtrate was concentrated to 50 cc. and poured into 3 volumes of ethyl alcohol. The product was reprecipitated three times by dissolving in 50 cc. of water and pouring into 200 cc. of absolute ethyl alcohol. Yield 3.1 gm. This amorphous barium salt could not be induced to crystallize. A sample of this substance (when dried at  $80^{\circ}$  over sulfuric acid) had a composition agreeing with that calculated for the barium salt of a pentose monophosphoric acid.

7.066 mg. substance: 42.710 mg. ammonium phosphomolybdate

39.229 " " : 24.910 " BaSO<sub>4</sub>

C<sub>5</sub>H<sub>9</sub>O<sub>5</sub>PBa. Calculated. P 8.49, Ba 37.6

365.45 Found. " 8.77, " 37.3

The air-dried material had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.99^{\circ} \times 100}{1 \times 5.28} = -18.8^{\circ} \text{ (in 0.1 N HCl)}$$

A portion of the crude barium salt was dissolved in water and the barium removed quantitatively with sulfuric acid. Brucine in methyl alcohol was added to the solution until just alkaline to litmus. The solution was concentrated to dryness and the product crystallized by dissolving in warm absolute methanol

and then cooling. The substance was recrystallized three times from absolute methanol and this pure material had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-2.24^\circ \times 100}{2 \times 2.304} = -48.6^\circ \text{ (50\% aqueous pyridine)}$$

The substance had a composition agreeing with that calculated for the dibrucine salt of a pentose monophosphoric acid.

12.010 mg. substance: 25.190 mg. ammonium phosphomolybdate

7.790 " " : 0.363 cc. N<sub>2</sub> (26° at 750 mm.)

C<sub>51</sub>H<sub>63</sub>O<sub>16</sub>N<sub>4</sub>P. Calculated, P 3.04, N 5.5; found, P 3.04, N 5.3





# A STUDY ON KIMMELSTIEL'S PROCEDURE FOR TITRIMETRIC CEREBROSIDE DETERMINATION, WITH DESCRIPTION OF AN IMPROVED TECHNIQUE\*

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Attempts to determine cerebrosides in a lipid mixture by hydrolysis with acid and titration of the liberated galactose were first made by Noll in 1899 (4), using Bertrand's copper reagent. An essentially similar technique was later employed by Winterstein and Hirschberg (8). In both procedures the total amount of reducing substance found after hydrolysis was considered to be galactose, as no reduction of the copper reagent was observed by Noll by the unhydrolyzed brain extract.

In a study on the reduction of Somogyi's improved copper reagent (7) by aqueous suspensions of the residues of alcoholic brain extracts the author was unable to confirm the findings of Noll, as in all the samples analyzed a definite reduction of the copper reagent was observed without hydrolysis. The cerebroside values observed by Noll, and by Winterstein and Hirschberg, therefore appear to be too high.

A notable improvement in the technique of analysis was introduced by Kimmelstiel (2) in 1929. In this procedure (No. I) the hydrolysis with acid was maintained, but the initial reduction values of the lipid mixture were subtracted from the values found after hydrolysis. The sugar determinations were performed by the ferricyanide method of Hagedorn and Jensen (1), the conditions for the reduction of the ferricyanide reagent by the galactose being carefully established. The significance of considering the

\* An abstract of the paper was read before the Eighteenth Medical Scandinavian Congress at Helsingfors, June, 1937. The investigation was aided by grants from the P. Carl Petersen Foundation and the Ella Sachs Plotz Foundation.

initial reduction of the lipid mixture in extracts from organs is obvious from figures given in Table III of Kimmelstiel's paper (2), the initial reduction value of the brain extract examined amounting to nearly half of the reduction value after hydrolysis.

Shortly after the publication of this method Kimmelstiel (3), however, introduced a modification of the technique (Procedure II), which appears to have made the procedure considerably less

TABLE I

*Effect of Zinc Precipitation on Reduction Values (Hagedorn-Jensen Procedure) of Unhydrolyzed Aqueous Lipid Emulsions from Alcoholic Brain Extracts*

The results are expressed in terms of mg. of galactose. All analyses, including blank determinations, were made in duplicate.

Source of brain material	Untreated samples	After zinc precipitation
Man.....	0.073	0.020
Hog.....	0.087	0.016
Codfish.....	0.175	0.020
Plaice.....	0.075	0.004
Flounder.....	0.027	0.000

TABLE II

*Effect of Zinc Precipitation on Reduction Values (Hagedorn-Jensen) of Hydrolyzed Lipid Samples from Alcoholic Brain Extracts*

The results are expressed in terms of mg. of galactose. All analyses, including blank determinations, were made in duplicate.

Source of brain material	Untreated samples	After zinc precipitation
Man.....	0.176	0.089
Hog.....	0.133	0.098
Codfish.....	0.250	0.093
Plaice.....	0.110	0.062
Flounder.....	0.078	0.045

accurate. The change of technique consisted in a precipitation with zinc hydroxide of that portion of the unhydrolyzed lipid sample which was used to determine only the initial reducing power. The purpose of this precipitation was to remove from the lipid emulsions any unsaturated fatty acids, which might cause too high initial reduction values, whereas no such acids were present in the water-clear, filtered, hydrolyzed samples.

As demonstrated by Kimmelstiel, and confirmed by the author in the present study (see Table I), such precipitation actually causes a considerable decrease of the initial reduction values, and thereby increases the figure for the cerebroside content calculated by subtracting the initial reduction from the reduction observed after hydrolysis. The fact, however, appears to have been overlooked that a large proportion of non-lipid reducing substances is likewise removed by zinc precipitation. These substances are also present in the hydrolyzed samples. The omission, therefore, of subjecting the hydrolyzed samples to zinc precipitation will result in the finding of too high cerebroside values.

The magnitude of error introduced by this omission is evident

TABLE III  
*Comparison of Cerebroside Determinations*

The values are expressed in terms of mg. of galactose. All analyses, including blank determinations, were made in duplicate.

Source of brain material	Kimmel- stiel's original Procedure I	Kimmel- stiel's modi- fied Pro- cedure II	Author's procedure	Values by author's pro- cedure in per cent of values by Kimmel- stiel's Pro- cedure II
Man .....	0.103	0.153	0.069	45
Hog .....	0.046	0.117	0.082	70
Codfish .....	0.075	0.230	0.073	32
Plaice .....	0.035	0.106	0.058	55
Flounder .....	0.051	0.078	0.045	58

from the figures in Tables II and III, which include analyses on alcoholic brain extracts from various species. Thus the cerebroside values found after zinc precipitation of both the hydrolyzed and unhydrolyzed samples amount to only one-third to seven-tenths of the figures obtained by Kimmelstiel's Procedure II. For the sake of completeness cerebroside values obtained by Kimmelstiel's original procedure (No. I) are also included in Table III.

Although the cerebroside values found after zinc precipitation of both the unhydrolyzed and hydrolyzed samples must be closer to the true cerebroside values than those obtained by Kimmelstiel's Procedure II, it still remains uncertain whether the figures represent the correct cerebroside values of the lipid extracts.

As the zinc precipitation of the interfering substances is not complete, the possibility exists, as also mentioned by Kimmelstiel, that erroneous values may be obtained through a change of the reduction value of these substances during the acid hydrolysis. Thus creatine under these conditions will be changed quantitatively to creatinine, which causes a reduction of the ferricyanide reagent twice that of creatine. As found by Kimmelstiel, however, the creatine content of alcoholic brain extracts is only small, but the possibility remains that other substances may be influenced through the hydrolysis in a similar or opposite way. The observed lack of agreement between results obtained by the original Kimmelstiel procedure (No. I) and those obtained after zinc precipitation of both unhydrolyzed and hydrolyzed samples are indicative of a change of the reducing ability of the interfering substances during the hydrolysis (see Table III).

It should not be overlooked either that the acid hydrolysis is performed on the unprecipitated samples, which tends to increase the possibility of error, a fact discussed above. Although a procedure including zinc precipitation of both unhydrolyzed and hydrolyzed samples thus probably gives only approximately correct figures, such procedure supposedly at the moment represents the most exact method for cerebroside estimation. As it has further been possible to simplify the technique, a detailed description of a modification of Kimmelstiel's procedure will be given below. This method gives accurate results with pure cerebroside samples, and permits the quantitative recovery of cerebroside added to alcoholic brain extracts.

#### *Modification of Kimmelstiel's Procedure for Cerebroside Determination*

The main changes in the technique besides the zinc precipitation of the hydrolyzed lipid samples described above, are the following. (1) In Kimmelstiel's method the hydrolysis is performed by heating the dry lipid residue with hydrochloric acid for 15 to 18 minutes in an oven at 112°. The samples are placed in special Jena flasks closed by ground glass stoppers, held in place by metal clamps. This somewhat complicated procedure has been replaced by 10 minutes heating in a boiling water bath, the samples being placed in ordinary Hagedorn tubes, covered by glass funnels, closed by a glass bead or provided with a fused lower tip (see Fig. 1).

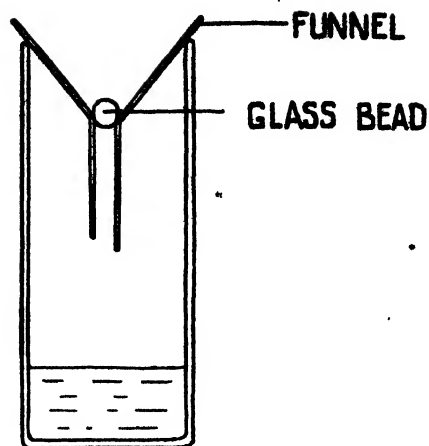


FIG. 1. Arrangement for acid hydrolysis of cerebroside sample

TABLE IV

*Analysis of Pure Cerebroside Samples by Author's Modification of Kimmelstiel's Procedure*

Cerebroside in sample		Deviation from theoretical	
Theoretical	Found		
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1.35	1.38	+0.03	+2.2
1.35	1.37	+0.02	+1.5
0.68	0.72	+0.04	+5.9
0.68	0.64	-0.04	-5.9
0.62	0.60	-0.02	-3.3
0.62	0.60	-0.02	-3.3
0.62	0.60	-0.02	-3.3
0.55	0.54	-0.01	-1.8
0.55	0.51	-0.04	-7.3
0.49	0.47	-0.02	-4.9
0.49	0.48	-0.01	-2.0
0.43	0.42	-0.01	-2.6
0.37	0.37	0.00	0.0
0.37	0.35	-0.02	-5.4
0.31	0.33	+0.02	+6.5
0.31	0.33	+0.02	+6.5

It was found in experiments with pure cerebroside samples that complete hydrolysis took place under these conditions (see Table IV). Furthermore, no loss of acid could be demonstrated as the result of the boiling. Exactly similar cerebroside values were found by extending the period of heating to 15 and 20 minutes, a fact which is in agreement with observations by Winterstein, who found no destruction of galactose after 30 minutes boiling with hydrochloric acid. (2) Phenol red has been substituted for methyl red as indicator in neutralization of the acid hydrolysate, because in reading the end-point of the thiosulfate titration phenol red interferes less than methyl red.

*Reagents—*

3 N hydrochloric acid solution.

Approximately 5 N sodium hydroxide.

Approximately 1 N hydrochloric acid.

0.1 N sodium hydroxide.

0.1 per cent aqueous solution of phenol red.

4.5 per cent solution of zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ).

Reagents for sugar determination, including washed cotton, as described by Hagedorn and Jensen.

*Procedure*

*Evaporation of Organic Solvent*—The samples, which should contain no more than 1 mg. of cerebroside, are pipetted into ordinary Hagedorn glasses, placed in a metal rack, and immersed in a boiling water bath for about 5 minutes for complete evaporation of the organic solvent.

*Determination of Initial Reduction Value (A)*—To the dry residue are added 5 cc. of water, after which the glass is covered with a funnel (Fig. 1) and heated for 10 minutes in a boiling water bath for emulsification of the lipids. After cooling, 0.5 cc. of the zinc sulfate solution is added, followed by exactly 2 cc. of the 0.1 N sodium hydroxide. Both these reagents may be added from burettes. The contents of the glass are then filtered through a small cotton filter into another Hagedorn glass provided with a mark at 17 cc. volume. A quantitative transfer is obtained through washing with three successive portions of 1 to 2 cc. of distilled water, which are likewise passed through the cotton filter. The sample is then made up to 17 cc. volume with distilled water,

2 cc. of the ferricyanide reagent are added, and the remaining part of the analysis performed as described by Hagedorn and Jensen.

A blank determination on the initial reduction procedure (B) is performed by carrying through the above analysis, including the zinc precipitation, with water instead of the lipid emulsion.

*Determination of Total Reduction Value (C)*—To the dry residue are added 4 cc. of 3 N hydrochloric acid from a burette. The glass

TABLE V  
Table for Calculation of Galactose Values from Thiosulfate Used in Titration (from Kimmelstiel (2))

Cc. 0.005 N thio- sulfate	Hundredths of 1 cc. 0.005 N thiosulfate									
	0	1	2	3	4	5	6	7	8	9
0.1	443	440	438	435	433	430	428	425	423	420
0.2	417	415	413	410	407	404	402	400	398	394
0.3	392	390	387	384	382	379	377	374	372	368
0.4	367	364	362	359	356	354	351	349	346	342
0.5	340	338	336	333	330	327	325	322	320	318
0.6	316	314	311	308	306	304	302	299	297	294
0.7	292	290	288	285	283	280	278	275	273	271
0.8	269	266	264	261	259	256	254	252	249	247
0.9	244	242	240	237	235	232	230	228	226	224
1.0	221	218	216	214	211	209	207	204	202	200
1.1	198	195	193	190	188	186	184	181	179	177
1.2	174	172	170	167	165	162	160	158	155	153
1.3	151	148	146	144	142	140	138	135	133	131
1.4	129	126	124	122	120	118	115	113	111	109
1.5	106	104	102	100	98	96	94	92	89	87
1.6	85	83	80	78	76	74	71	69	67	64
1.7	62	60	58	55	53	51	49	46	44	42
1.8	40	38	36	33	32	30	28	26	24	22
1.9	20	18	16	14	12	10	8	6	4	2

is covered with a funnel and heated for 10 minutes in a boiling water bath. The hydrolysis may be performed simultaneously with the heating of the samples for determination of the initial reduction value. After cooling, 1 drop of phenol red indicator is added, and the sample neutralized approximately by addition of 5 N sodium hydroxide till the appearance of the red color of the indicator, after which the sample is again brought back to the yellow color of phenol red with 1 N hydrochloric acid. The



sample is finally neutralized exactly by addition of 0.1 *N* sodium hydroxide. To the neutralized sample are added 0.5 cc. of the zinc sulfate solution and exactly 2 cc. of 0.1 *N* sodium hydroxide. Both these reagents may be added from burettes. The contents of the glass are then filtered through a small cotton filter into another Hagedorn glass, provided with a mark at 17 cc. volume. A quantitative transfer is obtained by washing with three successive portions of 1 to 2 cc. of distilled water, which are likewise passed through the cotton filter. The sample is then made up to 17 cc. with distilled water, 2 cc. of the ferricyanide reagent are added, and the remaining part of the analysis performed as described by Hagedorn and Jensen.

A blank value on the procedure of hydrolysis (*D*), including the effect of the salt on the ferricyanide reagent, is determined by performing the analysis with water instead of the lipid emulsion.

In all the titrations the soluble starch indicator should be added at the beginning of the titration, owing to the yellow color of the samples caused by phenol red in the acid solution. The factor of the 0.005 *N* thiosulfate solution used should be redetermined with each set of analyses.

*Calculation*—From the number of cc. of 0.005 *N* thiosulfate used the galactose values are obtained from the table given by Kimmelsstiel (2). For the sake of convenience this table is reprinted in the present paper (Table V).

Initial reduction value, *A* - *B*; total reduction value, *C* - *D*.

*Cerebroside Content of Sample*—(Total reduction value minus initial reduction value)  $\times$  4.6. The value 4.6 represents the factor for conversion of galactose figures into values of cerebroside.

#### EXPERIMENTAL

The results of analyses of samples of pure cerebroside are given in Table IV. The cerebroside was prepared from human brain according to Rosenheim (6) and Page (5), purified through extraction with ether, and recrystallized from alcohol and chloroform. The average analytical error was 4 per cent, the maximum error 7.3 per cent.

#### *Recovery of Cerebrosides Added to Alcoholic Extract of Human Brain*

	mg. per cent
Original cerebroside content of extract.....	31.7
After addition of 51.8 mg. cerebroside per 100 cc. extract....	82.0
Recovered .....	50.3
	or 97.0%

## SUMMARY

1. The procedure (No. II) of Kimmelstiel (3) for cerebroside determination was found to give too high values in analyses of alcoholic brain extracts from various species. The error was found to be due to the omission by Kimmelstiel of subjecting the hydrolyzed samples to precipitation with zinc hydroxide for removal of interfering reducing substances.

2. A modification of the method is described which includes this precipitation, and in which the procedure of hydrolysis is simplified. The method permits determination of pure cerebroside samples in amounts varying from 0.3 to 1.3 mg. with an average deviation between duplicates of 4 per cent, and likewise permits the quantitative recovery of added cerebroside. It still remains uncertain, however, whether the values found in analysis of organ extracts represent the true cerebroside values.

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# A MICROMETHOD FOR APPROXIMATE ESTIMATION OF LECITHIN, CEPHALIN, ETHER-INSOLUBLE PHOSPHATIDE, AND CEREBROSIDES IN PLASMA, RED BLOOD CELLS, AND TISSUES\*

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## Outline of Analysis

The lipids are extracted from plasma, red blood cells, and tissues with alcohol-ether, and subsequently isolated in petroleum ether as described by Kirk, Page, and Van Slyke (6). The phosphatides are precipitated from the petroleum ether extract with acetone and magnesium chloride according to Bloor (2), and the precipitate treated with moist ether which redissolves lecithin and cephalin. *Lecithin* is estimated in the moist ether extract by saponification with barium hydroxide and subsequent choline analysis according to Roman (11). *Cephalin* is calculated as the remaining ether-soluble phosphatide by subtracting the lecithin value from the total amount of ether-soluble phosphatide, estimated by gasometric carbon determination (Van Slyke, Page, and Kirk (15)) or by phosphorus analysis on the residue of an aliquot of the ether extract. The amount of *ether-insoluble phosphatide* is determined by phosphorus analysis of the fraction which does not dissolve in moist ether. *Cerebrosides* are estimated directly in the petroleum ether extract by the modification of Kimmelsiel's procedure (4) described in the preceding paper.

*Extraction of Lipids from Plasma, Red Blood Cells, and Tissues. Evaporation of Alcohol-Ether Extract; Resolution in Petroleum*

\* An abstract of the paper was read before the Eighteenth Medical Scandinavian Congress at Helsingfors, June, 1937. The investigation was aided by grants from the P. Carl Petersen Foundation and the Ella Sachs Plotz Foundation.

*Ether*—The extraction of lipids from plasma and red blood cells is carried out essentially as described in a previous paper (Kirk, Page, and Van Slyke (6)), 3 to 5 cc. of plasma and 2 to 5 gm. of red blood corpuscles being used for each extraction with 100 cc. of alcohol-ether. In the present study, however, the use of sand for transfer of the blood cells from the weighing bottle to the extraction flask was omitted, as a finely divided precipitate, the consistency of ground coffee, could be regularly obtained also in the absence of sand if the extraction flask was shaken frequently and vigorously during addition of the alcohol-ether.

For extraction of lipids from tissues the technique previously described was followed in detail.

The evaporation of the alcohol-ether extract below 60° was carried out somewhat more conveniently than previously described by leaving the beakers overnight on a metal shelf placed over a radiator. The lipids of the residue were redissolved by repeated extractions with portions of petroleum ether, which were afterwards passed through a porcelain filter and made up to a final volume of 50 cc. It was found that complete extraction of the lipids could be obtained by this volume of petroleum ether.

*Procedure for Determination of Lecithin, Cephalin, and Ether-Insoluble Phosphatide*—The principles for determination of these phosphatides are outlined above. For the sake of convenience the procedure of Bloor for phosphatide precipitation and Roman's titrimetric choline procedure will be included in the description.

*Reagents*—

*For Phosphatide Precipitation and Resolution of Lecithin and Cephalin*—The reagents are described by Bloor (2).

*For Determination of Lecithin*—Saturated aqueous barium hydroxide solution; approximately 5 per cent sulfuric acid; 0.2 per cent alcoholic phenolphthalein solution; Roman's iodine-iodide reagent, containing 15.7 gm. of iodine and 20 gm. of potassium iodide in 100 cc. of water; ice water; chloroform; 0.005 N sodium thiosulfate.

*For Determination of Cephalin and Ether-Insoluble Phosphatide*—The reagents for gasometric carbon determination are described by Van Slyke, Page, and Kirk (15), and for digestion and gasometric phosphorus determination by Kirk (5).

*Precipitation of Phosphatides According to Bloor*—15 cc. of

petroleum ether extract are evaporated in a centrifuge tube by immersing the tube in a beaker containing about 1 inch of hot water.<sup>1</sup> As advised by Bloor, a glass stick with a capillary tube fused to the end is placed in the centrifuge tube during the evaporation to prevent too vigorous boiling. When the sample has been concentrated to 1 cc. volume, 7 cc. of acetone and 3 drops of magnesium chloride solution are added, and the tube left standing for 10 minutes for precipitation of the phosphatides. During this period the sample is frequently stirred with the glass rod, which is also used for rubbing the sides of the centrifuge tube. After centrifugation for 5 minutes at approximately 2000 revolutions per minute the clear supernatant fluid is poured off. The precipitate and the sides of the tube are washed once with one 3 cc. portion of acetone, which is likewise decanted off after centrifugation. Finally the remaining traces of acetone are removed by sweeping through a current of air.

*Resolution of Lecithin and Cephalin from Phosphatide Precipitate*—To the residue in the centrifuge tube are added 5 cc. of moist ether and the precipitate is carefully stirred up by means of the stirring rod. By this procedure a great portion of the precipitate goes into solution. After 5 minutes the tube is centrifuged for 5 minutes at 2000 R.P.M., after which the clear, supernatant, moist ether is poured into another 15 cc. centrifuge tube, calibrated at 0.1, 0.2, 0.3, 5.0, and 10.0 cc. volume. The precipitate is again treated with 3 cc. of moist ether, which after centrifugation is added to the first portion of ether. The volume is then made up to exactly 10 cc. with ether, the sample is mixed by stirring, and two 1 cc. samples pipetted off for estimation by combustion of the total phosphatide content of the extract (see below).

*Determination of Lecithin. Saponification of Lecithin; Neutralization of Saponified Sample*—The remaining 8 cc. of moist ether are evaporated gently by placing the centrifuge tube in a dry beaker on a steam bath.<sup>1</sup> To the dry residue 3 cc. of saturated barium hydroxide solution are added, after which the tube is tightly corked and left immersed in the steam bath for 30 minutes. After cooling, 1 drop of phenolphthalein indicator is added, and the sample neutralized by addition of 5 per cent sulfuric acid.

<sup>1</sup> The evaporation may also be accomplished by leaving the tubes at room temperature for about 24 hours.

The volume is then made up to the 5 cc. mark with distilled water, after which the tube is centrifuged for 2 minutes at 1000 R.P.M. for sedimentation of the barium sulfate, the volume of which is noted for calculation of the volume of the fluid phase. The supernatant fluid is usually water-clear. In case a slight turbidity should be present, this can be removed by adding another drop of sulfuric acid and repeating the centrifugation.

*Determination of Choline According to Roman*—2 cc. of the supernatant fluid, representing approximately 6 cc. of the petroleum ether extract, are pipetted into a centrifuge tube and 0.6 cc. of the iodine reagent added. In case larger amounts of choline are present, this addition results in the occurrence of a marked turbidity. The tube is then centrifuged for 10 minutes at 2000 R.P.M., after which the supernatant fluid and successive 2 to 3 cc. portions of ice-cold wash water are passed with the use of suction through a small porcelain filter (Jena G3) to secure any particles of the precipitate which might be floating in the supernatant fluid. The washings of the centrifuge tube are continued until the wash water is free of color, three washings usually sufficing. As the precipitate is slightly soluble in ice water, the washings, as also emphasized by Roman, should be carried out as rapidly as possible. At every step of the analysis great care must be taken to prevent evaporation of iodine from the precipitate; this is best avoided by never allowing air to be sucked through the filter during the filtration. In the present study the use of a porcelain filter was found obligatory for obtaining accurate results, although this precaution is recommended by Roman only in analysis of large choline samples.

After completion of the washings 1 to 2 cc. of chloroform is added to the precipitate in the centrifuge tube and about 0.5 cc. to the cup of the porcelain filter. The latter portion is afterwards transferred to the centrifuge tube by suction. The iodine compound dissolves in the chloroform with a red color.

The titration of the iodine is performed with 0.005 N sodium thiosulfate, for which a Bang microburette with a fine delivery tip is used. During the titration the contents of the tube are shaken vigorously at frequent intervals after insertion of a cork stopper. The titration is continued till the disappearance of the red color. The determination of the end-point presents no difficulties if the tube is viewed against a white background.

*Calculation*—Choline content of sample, cc. of 0.005 N sodium thiosulfate used  $\times$  0.067. Lecithin content of sample, choline value  $\times$  6.68.

*Determination of Cephalin*—1 cc. samples of the moist ether extract are pipetted into the combustion tubes devised by Van Slyke, Page, and Kirk (15). After evaporation of the ether the residue is subjected to gasometric carbon determination by combustion. The total lecithin + cephalin value is estimated by multiplication of the carbon value with 1.52, the carbon content of lecithin and cephalin being approximately 66 per cent. The cephalin is estimated by subtracting the lecithin from the combined lecithin + cephalin.

The amount of lecithin + cephalin may also be determined by phosphorus analysis of an aliquot of the moist ether extract. This procedure involves preliminary digestion with acid but may be employed in the absence of facilities for gasometric analyses.

*Determination of Ether-Insoluble Phosphatide*—The residue in the centrifuge tube is dissolved in 95 per cent alcohol and transferred to a Pyrex tube for digestion. The transfer can be most conveniently accomplished by adding 5 cc. of alcohol and bringing the precipitate into solution or emulsion by stirring with a glass rod. An aliquot of the alcoholic solution, usually 4.5 cc., can then be pipetted off for phosphorus determination. The digestion of the sample and the subsequent gasometric phosphorus analysis are performed as described by Kirk (5).

If less accuracy is required than is obtainable by the gasometric method, the following procedure, the error of which rarely exceeds 2 per cent, may be used with advantage. After digestion and neutralization the samples are precipitated with strychnine molybdate, transferred to a Pregl filter, and washed with dilute nitric acid and distilled water as described by Kirk (5). After washing, the precipitate is redissolved in 1 N sodium hydroxide. Three successive portions of 1 cc. each are added to the cup of the filter and transferred by suction to a 50 cc. flask. The filter is rinsed with two 1 cc. samples of distilled water, which are likewise transferred to the flask for color development according to Tisdall (14).

*Calculation*—Phosphatide content of sample, P  $\times$  25.8.

*Determination of Cerebrosides*—The cerebroside determination is carried out as described in the preceding paper, 3 cc. samples



of the petroleum ether extract being used both for determination of the initial reduction value and the reduction after hydrolysis. Before the sample is pipetted out, the flasks containing the petroleum ether extract should be shaken carefully to bring any precipitated cerebroside into emulsion.

#### DISCUSSION

*Lecithin*—For determination of lecithin the procedures most frequently used have been based on choline estimation after saponification of the lipid mixture. As, however, sphingomyelin also yields choline on saponification, a separation of lecithin from sphingomyelin is necessary, a fact which has frequently not been considered. For choline determination various methods have been employed, especially precipitation as the platinum chloride or iodine compound. Another principle for choline determination was introduced in 1931 by Lintzel and Fomin (7) and by Lintzel and Monasterio (8), the choline being destroyed by boiling with strong alkali and the liberated trimethylamine estimated by titration. The applicability of the platinum chloride precipitation is limited by the fact that several other substances enter into the reaction. The iodine precipitation appears to be more specific, but until recently accurate estimation of minute amounts of choline was not possible by this procedure, owing to a measurable solubility of the choline-iodine compound in the precipitant. In 1930 Roman (11), however, succeeded in establishing the conditions for quantitative precipitation with iodine, thus making the method applicable to determination of amounts of choline as small as 0.005 to 5 mg., the analytical error of the procedure usually not exceeding 5 per cent. In view of the simplicity of the technique the iodine precipitation appears preferable to the procedure of Lintzel and Monasterio, which, furthermore, requires choline samples as large as 2 to 5 mg. for accurate results.

A possible limitation in the applicability of the method is, however, afforded by the fact that, besides choline, creatinine is precipitated by the iodine reagent. To test the significance of this inclusion aqueous emulsions of the residues of a series of petroleum ether extracts from human plasma and red blood cells were treated with picric acid and sodium hydroxide. In none of the samples examined was any color development observed.

The possibility of inclusion in the analysis of choline derived from sphingomyelin represented another likely source of error due to the intersolubility of the various phosphatides. As will be demonstrated below, inclusion of sphingomyelin in significant amounts hardly takes place under the conditions of the method (see Table III).

In view of the fact, however, that petroleum ether extracts from blood and tissues contain large amounts of nitrogenous substances other than phosphatides (Kirk, Page, and Van Slyke (6), Van Slyke, Page, Kirk, and Farr (16)), the presence in the saponified sample of other substances than choline precipitable by the

TABLE I  
*Analysis of Choline Chloride Standard Solutions by Method of Roman*

Choline in sample	0.005 N thiosulfate used	Choline found	Deviation from theoretical	
mg.	cc.	mg.	mg.	per cent
0.0484	0.707	0.0475	-0.0009	-1.9
0.0484	0.700	0.0469	-0.0015	-3.1
0.0968	1.460	0.0980	+0.0012	+1.2
0.0968	1.460	0.0980	+0.0012	+1.2
0.1039	1.500	0.1010	-0.0029	-2.9
0.1039	1.500	0.1010	-0.0029	-2.9
0.1936	2.920	0.1960	+0.0024	+1.2
0.1936	2.920	0.1960	+0.0024	+1.2
0.2078	2.990	0.2004	-0.0074	-3.6
0.2078	3.020	0.2080	+0.0002	+0.2

iodine reagent cannot be definitely excluded. That the inclusion of such interfering substances hardly can be considerable is seen from the fact that in analyses of 104 samples of human plasma and erythrocytes the lecithin values calculated from the choline determinations never were found to exceed the value of total ether-soluble phosphatide, estimated by gasometric carbon determination on the moist ether extract.

Results obtained by analysis of standard choline chloride solutions by the method of Roman are given in Table I. Experiments were further performed to ascertain whether any destruction of choline occurred under the conditions of the saponification. As the following figures show, no measurable loss of choline could be

demonstrated even after a period of saponification twice that used for hydrolysis of lecithin.

	<i>mg.</i>
Original choline content of sample.....	0.266
After saponification for $\frac{1}{2}$ hr.....	0.267
Original choline content of sample.....	0.350
After saponification for 1 hr.....	0.344

These findings are in good agreement with observations by Gulewitsch (3) and by MacLean (9).

TABLE II  
*Determination of Lecithin by Titrimetric Choline Estimation after  
Saponification with Barium Hydroxide*

Lecithin in sample subjected to saponification	Choline found*	Lecithin found (choline $\times$ 6.68)	Deviation from theoretical	
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
0.500	0.071	0.476	-0.024	-4.8
0.500	0.069	0.468	-0.032	-6.8
1.004	0.142	0.951	-0.053	-5.3
1.004	0.156	1.041	+0.037	+3.7
2.008	0.292	1.950	-0.058	-2.9
2.008	0.305	2.060	+0.052	+2.6
2.008	0.298	1.980	-0.028	-1.4
2.008	0.296	1.974	-0.034	-1.7
3.012	0.462	3.080	+0.068	+2.3
3.012	0.470	3.136	+0.124	+4.1
5.020	0.794	5.280	+0.260	+5.2
5.020	0.776	5.180	+0.160	+3.2

\* The choline determinations were performed on 2 cc. aliquots of the saponified sample, the fluid volume after saponification being approximately 5 cc. (see description of the method in the text).

The adequacy of a 30 minute saponification period was confirmed by analyses of standard lecithin samples, and by comparing results obtained by this procedure with results obtained after a longer period of saponification. Thus after saponification of a lecithin sample for 30 minutes 0.092 mg. of choline was yielded, while aliquot samples after 60 and 90 minutes gave values of 0.094 and 0.091 respectively, these differences being within the limits of experimental error.

The accuracy of the method was tested by analysis of samples

of a pure lecithin-cephalin mixture, prepared from egg according to MacLean and MacLean (10), the cephalin content of which had been carefully assayed by gasometric amino nitrogen determination according to Kirk, Page, and Van Slyke (6). In these experiments the phosphatide sample, containing no sphingomyelin, was subjected directly to saponification. The results presented in Table II show an analytical error rarely exceeding 5 per cent. Similar lecithin values were, however, obtained if previous to the

TABLE III

*Analysis of Choline Content of Standard Lecithin-Cephalin Samples and of Same Samples after Addition of Known Amounts of Sphingomyelin*

Sample	Treatment	Choline found*
mg.		mg.
2.08. Cephalin } 1.72. Lecithin }	Saponified directly	0.255
Same	Same	0.260
	Saponification after Bloor, precipitation and resolu- tion in moist ether	0.258
	Same	0.256
2.08. Cephalin 1.72. Lecithin		0.254
3.10. Protagon (containing 0.38 mg. sphingomyelin)		
2.08. Cephalin 1.72. Lecithin		0.245
4.90. Protagon (containing 0.60 mg. sphingomyelin)		

\* Theoretical choline value for lecithin content of sample, 0.256 mg.

saponification the phosphatides were precipitated with acetone and magnesium chloride according to Bloor and the precipitate redissolved in moist ether, correction being made for the small amount of phosphatide (approximately 2 per cent) which escapes precipitation by this procedure. This observed agreement confirms the statement by Bloor that lecithin under the conditions of the method is completely soluble in moist ether (see Table III).

*Cephalin*—In a previous publication the conditions for deter-

mination of cephalin by gasometric amino nitrogen analysis were outlined (Kirk, Page, and Van Slyke). In the same paper it was, however, emphasized that the petroleum ether extracts of blood and tissues contain considerable amounts of non-phosphatide amino nitrogen. The nature of these nitrogenous constituents was later subjected to a more detailed study (Van Slyke, Page, Kirk, and Farr), and it was found that part of the amino nitrogen could be removed by shaking with acidified water. As such procedure was claimed by Schmitz and Koch (12) to remove quantitatively the non-phosphatide amino nitrogen, this promised a means for accurate cephalin determination by direct analysis. In accordance with the technique described by Schmitz and Koch petroleum ether extracts of human plasma and red blood cells were shaken with 0.5 volume of 0.01 *N* sulfuric acid containing 20 per cent of magnesium sulfate. It was found that up to 50 per cent of the amino nitrogen of the extract could be removed by 4 minutes shaking, whereas continued shaking up to 20 minutes caused no additional removal. Furthermore, in agreement with the statements by Schmitz and Koch, such treatment was found to remove no phosphatide, as evidenced by gasometric amino nitrogen analysis of a standard cephalin solution in petroleum ether before and after 20 minutes shaking with the acid reagent. The statements by Schmitz and Koch on the quantitative removal of non-phosphatide amino nitrogen could, however, not be confirmed, as the cephalin values calculated from gasometric amino nitrogen analyses usually were found to exceed greatly the cephalin values obtained by the procedure described in this paper, and occasionally even the total phosphatide values determined by phosphorus analysis in the petroleum ether extracts. The results of analysis of 62 samples of petroleum ether extracts by the two procedures are given in Fig. 1.

The indirect procedure for cephalin determination suggested in the present publication therefore probably at the moment represents the least inaccurate method for such analysis. An error in the determination is introduced by the fact that the moist ether extract contains nitrogenous substances other than the phosphatides. This is evidenced by the not infrequent finding of an amino nitrogen concentration of the moist ether extract higher than that calculated from the value of total ether-soluble phosphatide. The

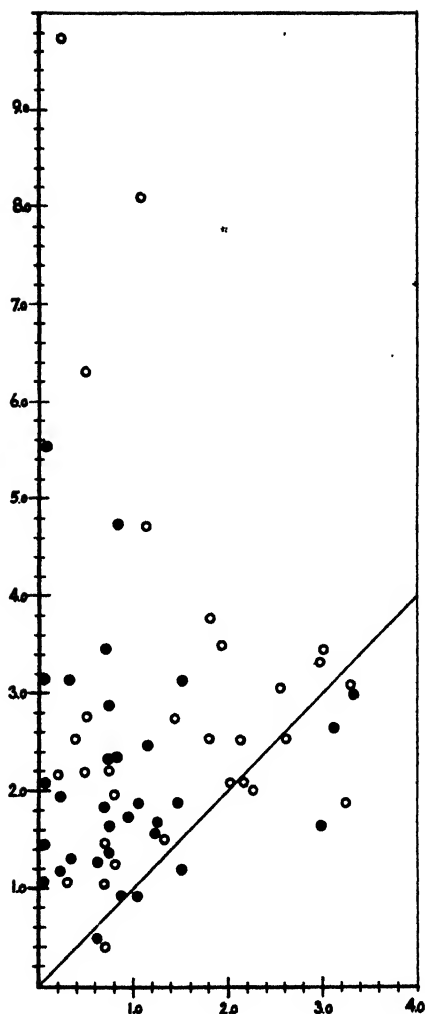


FIG. 1. Comparison of gasometric amino nitrogen values (abscissa) determined by the procedure of Kirk, Page, and Van Slyke (6) after shaking the petroleum ether extract with the sulfuric acid-magnesium sulfate reagent, and amino nitrogen values (ordinate) calculated from the cephalin figures determined by the procedure described in the text. The results are expressed in mg. per cent of amino nitrogen. ○ plasma analyses; ● red blood cell analyses.

presence of carbohydrates in combination with the monoaminophosphatides (Bing (1)) should also be considered. Inclusion of such and other substances in the ether extract will tend to give too high cephalin figures, and may explain why in rare instances the cephalin values calculated in the manner described exceed the values determined by gasometric amino nitrogen analysis after the petroleum ether extract is shaken with acidified magnesium sulfate solution (see the readings below the diagonal line in Fig. 1). The error might be avoided by determining the amount of ether-soluble phosphatide through phosphorus estimation instead of carbon analysis.

*Ether-Insoluble Phosphatide*—According to the knowledge of the author no procedure has been suggested for determination of sphingomyelin in a mixture of phosphatides.<sup>2</sup> The possibility of a method for such determination presented itself through the demonstration by Kirk, Page, and Van Slyke that the phosphorus and nitrogen content of the residue insoluble in moist ether obtained after acetone precipitation of the phosphatides from human blood corresponded to that of a diaminomonomophosphatide. In order to study further the behavior of sphingomyelin under the conditions of acetone precipitation a sample of protagon was prepared from human brain, and the sphingomyelin content estimated by phosphorus analysis. This preparation was found to yield choline under the conditions employed for saponification of lecithin. After precipitation of a sample containing 0.5 mg. of sphingomyelin (4.2 mg. of protagon) with acetone and magnesium chloride the precipitate was treated with several portions of moist ether. The moist ether extracts after saponification were found to give a negative choline reaction. Similar experiments were performed, in which protagon was added to a standard sample of lecithin and cephalin. As is seen from Table III (lower section), such addition did not influence the choline values found in analysis of the moist ether extract.

<sup>2</sup> Since the completion of this study the author has become acquainted with the interesting publication by Thannhauser and Setz (13), in which a separation between monoaminophosphatide and diaminophosphatide is accomplished by precipitating the latter as reineckate. A comparison of results obtained by the method of Thannhauser and Setz and by the present procedure will be given in the following paper.

In addition to the theoretical recovery by the procedure of lecithin, cephalin, and sphingomyelin in analysis of standard phosphatide samples (see Table IV), several other observations indicate that the separation of sphingomyelin and lecithin-cephalin is practically complete under the conditions of the Bloor procedure. Thus if the moist ether extract of phosphatides from human brain is concentrated and again subjected to precipitation with acetone and magnesium chloride, this precipitate redissolves clearly in moist ether without leaving anything but traces of organic material. Also, treatment of the original phosphatide precipitate from human blood with a third portion of moist ether was found to cause as little as 0.3 per cent of the total amount of

TABLE IV  
*Analysis of Mixtures of Standard Lecithin, Cephalin, and Sphingomyelin Samples*

	Theoretical	Found	
		I	II
	mg.	mg.	mg.
Lecithin .....	99.0	96.1	100.4
Cephalin .....	51.4	49.9	52.3
Sphingomyelin .....	34.7	38.0	33.0
Total phosphatide .....	185.1	184.0	185.7

residue left to go into solution. Finally, practically the same analytical results were obtained when the analysis was carried through with one-half of the volume of petroleum ether as when the usual volume was used for precipitation.

Although these observations show that sphingomyelin, if present, will be found in the phosphatide fraction insoluble in moist ether, it does not appear that this fraction consists of sphingomyelin only. Thus in analysis of forty-eight samples of human plasma and forty-nine samples of red blood cells more phosphorus was found in the phosphatide fraction insoluble in moist ether in forty-one of the plasma samples and thirty-seven of the erythrocyte samples than corresponded to the amount of iodine-precipitable substance yielded by saponification of an



aliquot of the residue with saturated barium hydroxide for 30 minutes. In reference to these results it should be stated, however, that the conditions for saponification of sphingomyelin appear to be more difficult to control than those for saponification of lecithin.

*Cerebrosides*—For discussion of procedures for cerebroside determination reference is made to the preceding paper. The lack of agreement observed between results obtained by Kimmelsiel's (4) procedure and by the modified method introduced by the author was confirmed in the present study in analysis of extracts of human plasma and red blood cells.

It was further demonstrated that the extraction of cerebrosides by petroleum ether from the residue of the alcohol-ether extract was complete, as subsequent extractions with tetralin and pyridine failed to yield any cerebroside.

#### SUMMARY

A procedure is described for approximate determination of lecithin, cephalin, ether-insoluble phosphatide, and cerebrosides in plasma, red blood cells, and tissues. The principles of analysis are outlined and the possible sources of error discussed.

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# **THE CONCENTRATION OF LECITHIN, CEPHALIN, ETHER-INSOLUBLE PHOSPHATIDE, AND CEREBROSIDES IN PLASMA AND RED BLOOD CELLS OF NORMAL ADULTS\***

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Owing to the lack of suitable micromethods few data are available in the literature on the concentration in the blood of the individual phosphatides and of cerebroside. The few lecithin and cephalin blood values reported are invalidated by the fact that inclusion of sphingomyelin in the analyses has not been considered. The presence of cephalin and sphingomyelin in the red blood cells of man and sheep was demonstrated by Bürger and Beumer (1) in 1913. The occurrence of a diaminophosphatide in human blood was confirmed by Kirk, Page, and Van Slyke (4) in 1934 by analysis of the phosphatide fraction insoluble in moist ether after acetone precipitation of the phosphatides. In 1936 Thannhauser and Setz (6) published the results of analyses of ten normal human sera, in which the diaminophosphatide was separated from the monoaminophosphatide by precipitating the former as the reineckate. The figures show an average diaminophosphatide concentration of 128 mg. per cent, the values ranging between 86 and 220 mg. per cent. From the data reported in the literature it appears doubtful, however, whether lecithin is a normal constituent of the red blood corpuscles, as Bürger and Beumer (1) (1913) and Haurowitz and Sládek (2) (1928) failed to detect any choline after saponification of the ether-soluble phosphatide fraction.

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In the present study the concentrations of the individual phosphatides and of cerebrosides were determined in twenty samples of normal human blood, the analyses being performed on heparinized plasma and isolated red blood cells by the method of the author

TABLE I

*Concentration of Individual Phosphatides and of Cerebrosides in Normal Human Plasma*

Subject	Sex	Age	Total fat	Total phosphatide	Ether-insoluble phosphatide	Lecithin	Cephalin	Cerebrosides
			mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
A. L.	M.	19	385	115	24	14	77	90
E. L.	"	19	421	148	38	18	92	0
E. S.	"	20	369	54	17	4	33	
C. H.	"	21	424	109	18	17	74	0
E. A.	F.	21	532	235	58	5	172	
E. K.	M.	21	393	122	55	20	47	73
P. F. H.	"	21	455	115	82	15	18	
H. L.	"	21	507	182	56	30	96	17
P. H.	"	21	548	155	22	37	96	11
E. P.	"	21	585	128	91	3	34	2
E. J.	"	22	479	108	32	64	12	78
A. N.	F.	22	465	133	45	29	55	
J. H.	M.	22	465	212	128	11	73	167
K. P.	F.	24	457	86	44	4	38	
B. H.	M.	24	775	205	126	18	61	7
P. H. P.	"	25	543	92	33	18	41	
E. M.	F.	27	487	195	156	5	34	19
A. N.	"	28	902	192	22	0	170	
M. K.	M.	28	373	153	91	9	53	83
E. K.	"	31	620	161	16	63	79	0
Average.....			559	145	58	19	68	
Total phosphatide, %.....					40	13	47	

(3) described in the preceding paper. The blood samples were drawn in the morning during fasting.

The results, presented in Tables I and II, show a considerable variation in the concentration of the individual phosphatides in the different subjects studied. The *total phosphatide* values of

plasma were somewhat lower than those found in a previous study (5) on a group of normal American individuals. In the case of the red blood cells no comparable data have previously been published, as no analyses on isolated erythrocytes of normal adults are

TABLE II

*Concentration of Individual Phosphatides and of Cerebrosides in Normal Human Erythrocytes*

Subject	Sex	Age	Total fat	Total phosphatide	Ether-insoluble phosphatide	Lecithin	Cephalin	Cerebrosides
			mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
A. L.	M.	19	297	189	51	29	109	0
E. L.	"	19	338	165	28	23	114	59
E. S.	"	20	469	248	25	43	180	
C. M.	"	20	447	228	22	42	164	12
C. H.	"	21	388	272	17	50	203	59
E. A.	F.	21	416	260	64	56	140	
E. K.	M.	21	386	226	37	48	141	74
P. F. H.	"	21	196	78	30	5	43	
H. L.	"	21	595	259	18	65	176	51
E. P.	"	21	304	111	103	5	3	45
E. J.	"	22	332	278	21	95	162	
A. N.	F.	22	742	174	16	5	123	
J. H.	M.	22	384	216	71	8	137	113
K. P.	F.	24	457	86	44	4	38	
B. H.	M.	24	298	100	91	3	6	55
H. L. L.	"	24	595	259	18	65	176	51
E. M.	F.	27	432	297	157	4	136	33
A. N.	"	28	381	273	22	60	191	
M. K.	M.	28	340	199	82	20	97	82
E. K.	"	31	180	26	13	3	10	26
Average.....			400	196	47	32	117	51
Total phosphatide, %.....					24	16	60	

available. As seen from Tables I and II the *lecithin* concentration both in plasma and in the red blood cells was often found to be very low, amounting to only a few mg. per cent, whereas the *cephalin* fraction, at least in the cells, usually constituted the major part of the phosphatides. These observations are in good agree-

ment with the findings reported by Bürger and Beumer. The average concentration of ether-insoluble phosphatide of plasma found in the present study was, however, definitely lower than the diaminophosphatide values reported by Thannhauser and Setz in serum analyses. In considering this difference, the fact should be noted that the total phosphatide values found by the author amounted to only about one-half of those observed by Thannhauser and Setz, the average concentrations being respectively 145 and 244 mg. per cent. If, therefore, the percentage of the total phosphatide value is calculated, a fair agreement will be seen, the figures being respectively 40 and 52 per cent. In the case of the red blood cells no comparable data are available, as the analyses of Thannhauser and Setz were performed on blood cell stroma, whereas the results presented in this paper were obtained on unwashed red blood cells, isolated by centrifugation.

The *cerebrosides* both in plasma and cells showed even greater variation in concentration than the phosphatides.

#### SUMMARY

A report is given on the concentration of lecithin, cephalin, the ether-insoluble phosphatide, and cerebrosides in the plasma and red blood cells of normal adults. The analyses were performed by a technique devised by the author.

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## PETROLEUM ETHER-SOLUBLE AND ETHER-SOLUBLE CONSTITUENTS OF GRAPE POMACE\*

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In continuation of research dealing with the identification of the constituents of the wax-like coatings of fruits, an account is now given of the separation and identification of such substances from the surface of the grape.

That the grape (*Vitis* spp.) has long been a subject of chemical investigation is evidenced by the compilation of such work by Wehmer (1). Little attention, however, has been paid to the nature and chemical composition of the wax-like coating of the skin or cuticle. Although it was stated as early as 1840 that the waxy coating of the grape amounted to 1 to 2 per cent of the fruit, it was not until 1892 that an attempt was made to determine the nature of the chemical constituents comprising this wax.

Etard (2) examined the carbon disulfide extract of dried white grape skins and concluded that it consisted principally of free fatty acids (85 per cent) together with a substance which he designated œnocarpol alcohol. Œnocarpol alcohol was obtained in the form of long needles melting at  $304^{\circ}$  and giving a specific rotation of  $[\alpha]_D = +60.8^{\circ}$  (ether). The product distilled completely at  $405^{\circ}$  with the formation of a balsam-like polyterpene having a composition corresponding to  $C_{36}H_{36}$ . Etard prepared a monoacetyl derivative of œnocarpol (m.p.  $215^{\circ}$ ) and a number of its metallic salts. On the basis of the results of combustions of the original substance and certain of its derivatives he assigned to œnocarpol alcohol the formula,  $C_{36}H_{39}(OH)_2 \cdot H_2O$ . By fractional crystallization of the crude wax with neutral solvents Etard

\* Food Research Division Contribution No. 352.

isolated a compound melting at  $272^{\circ}$  which he concluded was the palmityl ester, œnocarpol palmitate.

Shortly after Etard's work appeared Seifert (3) reported the results of a similar examination of the waxy coating from thirteen varieties of American grape hybrids. One of the products was obtained in the form of colorless, silky needles, melting at  $250-255^{\circ}$  and having a specific rotation of  $[\alpha]_D = +59.879^{\circ}$  (ethanol). After repeated recrystallization from ethanol the compound gave on analysis C 78.98, H 10.93, corresponding to the empirical formula  $C_{16}H_{16}O$ . The product gave an acetyl derivative melting at  $239^{\circ}$  and well defined metallic salts. On the basis of these results Seifert concluded the compound was a weak acid, containing a free hydroxyl group, and that its molecular formula was  $C_{20}H_{32}O_2$ . He therefore named the compound vitin in reference to its source.

The results of the present work indicate that Etard's œnocarpol alcohol and Seifert's vitin are in reality more or less pure oleanolic acid,  $C_{30}H_{48}O_3$ , melting at  $310-310.5^{\circ}$ . Oleanolic acid was isolated in 1908 from olive leaves by Power and Tutin (4) under the name of oleanol and assigned the formula  $C_{31}H_{50}O_3$ . Van der Haar (5) reinvestigated oleanol and concluded it was isomeric with ursolic acid and therefore should be designated oleanolic acid. The presently accepted formula for oleanolic acid,  $C_{30}H_{48}O_3$ , was established by the work of Dodge (6) on caryophyllin, of Wedekind and Schicke (7) on guagenin, and of Ruzicka and Furter (8) on the sapogenins from the sugar beet and *Viscum album*, which have been shown to be identical with oleanolic acid. Kuwada and Matsukawa (9) isolated the sapogenin present in the cuticle of grape leaves and identified it as ursolic acid, whereas we find the sapogenin present in the cuticle of the fruit is the isomeric oleanolic acid. Thus it appears that two closely related organs of the same plant may elaborate two different sapogenins.

Seifert also examined the non-resinous constituents present in grape wax. After saponification he obtained by solvent fractionation a considerable number of alcoholic and saturated fatty acid fractions. None of the fractions could be obtained pure but on the basis of analyses of partially purified fractions Seifert concluded the wax contained ceryl and myricyl alcohols and palmitic and cerotic acids. The present work indicates that the wax con-

tains glycerides of saturated and unsaturated acids, primary alcohols, hydrocarbons, and a sterol.

#### EXPERIMENTAL

The material used in this investigation consisted of air-dried pomace from Concord grapes (*Vitis labrusca*) which was supplied by The Welsh Grape Juice Company, Westfield, New York. The pomace consisted principally of skins to which adhered small amounts of fleshy material. After the pomace was ground to pass a sieve having 1 mm. openings, it was exhaustively extracted in a Sando (10) extractor with petroleum ether (b.p. 40–60°) and then with ethyl ether. 6 kilos of pomace yielded 240 gm. of petroleum ether extract and 203 gm. of ether extract, equivalent to 4.0 and 3.4 per cent, respectively, of the air-dried skins.

*Petroleum Ether Extract*—The residue from the petroleum ether extraction was a bluish black, wax-like solid at room temperature. The iodine number was 79.0.<sup>1</sup> A portion (132 gm.) of the crude wax was saponified with 1500 ml. of benzene-ethanol (1:4) solution containing 125 gm. of potassium hydroxide dissolved in 150 ml. of water. The mixture was refluxed for 7 hours, after which approximately three-fourths of the solvent was removed by distillation. The residue was poured into water and the resulting mixture digested on the steam bath. After digestion and cooling, the unsaponifiable matter was extracted with ether. The ethereal solution was washed with water and the ether removed by evaporation, whereupon there were obtained 54 gm. (40.9 per cent) of unsaponifiable matter.

The aqueous solution remaining after extraction with ether was filtered to separate the interfacial and suspended solids which amounted to 7.0 gm., or 5.3 per cent of the original wax. The aqueous filtrate was acidified and the fatty acids which floated to the surface were separated by siphoning off the underlying aqueous layer. The acids were washed by repetition of the process of heating with water and siphoning off the aqueous layer. The aqueous solutions were combined and shaken with ether to remove any further acids present. The acids previously separated were added to the ether solution which was digested with decolorizing

<sup>1</sup> Iodine numbers throughout the paper were determined by the Rosenmund and Kuhnhehn method (11).



carbon. After filtration and evaporation of the ether there were obtained 66.0 gm. (50.0 per cent) of mixed fatty acids.

*Glycerol*—The aqueous solution remaining after separation of the fatty acids was neutralized and evaporated nearly to dryness. After extraction of the residue with absolute ethanol containing a small amount of ether, the extract was clarified in the usual manner and concentrated on the steam bath. The residue (5.8 gm.) was a colorless, viscous liquid which gave all the usual tests for glycerol.

*Fatty Acids*—The mixed fatty acids (iodine number 92.9) were separated into saturated and unsaturated acid fractions by the lead salt-ether procedure as modified by Jamieson (12). The recovered saturated acids amounted to 16 gm. and the unsaturated acids to 46 gm. Since linolenic acid was found to be absent, the composition of the unsaturated fraction was calculated from the iodine value (124.3). This calculation indicated the presence of 37.7 per cent linoleic acid and 62.3 per cent oleic acid in the unsaturated acid fraction.

*Linoleic Acid*,  $C_{18}H_{32}O_2$ —The liquid fatty acids (46.0 gm.) were dissolved in anhydrous ether (460 ml.) cooled to  $-13^\circ$  and slightly more than the theoretical amount of bromine was added dropwise over a period of 2 hours. No hexabromide separated during the bromination. The excess bromine was destroyed with  $\beta$ -amylenes and the ethereal solution evaporated to dryness. The residue was extracted with petroleum ether and the extract allowed to stand until the next day. The crude tetrabromostearic acid was separated by filtration, dissolved in ether, and the ethereal solution treated with decolorizing carbon. The filtered solution was evaporated to dryness and the residue recrystallized from petroleum ether. The recovered tetrabromostearic acid (22 gm.) melted at  $114.0$ – $114.5^\circ$  and gave on analysis C 36.64, H 5.50.<sup>2</sup> Calculated for tetrabromostearic acid,  $C_{18}H_{32}O_2Br_4$ , C 36.00, H 5.38.

*Oleic Acid*,  $C_{18}H_{34}O_2$ —The petroleum ether filtrate remaining after removal of the tetrabromides was evaporated to a syrup and

<sup>2</sup>For all carbon and hydrogen determinations reported in this paper, the writers are greatly indebted to Mrs. Mildred S. Sherman, Fertilizer Investigations, Bureau of Chemistry and Soils, and Dr. W. T. Haskins, National Institute of Health, United States Public Health Service.

allowed to stand several weeks at low temperature. It was then treated with ice-cold petroleum ether which dissolved the dibromides, leaving behind an additional quantity of crystalline tetrabromide.

The petroleum ether solution was evaporated and the residue debrominated in the usual manner with zinc dust in alcohol solution. The debrominated acid was a faintly yellow oil which on standing overnight in a desiccator deposited a considerable quantity of fine white needles. Cold petroleum ether was added to the mixture and the undissolved crystalline material filtered off. The solid (5.0 gm.) melted at 72°. It was not appreciably soluble in petroleum ether, chloroform, or acetone, and only slightly so in ethanol. It was, however, readily soluble in ether. The recrystallized product could be titrated with alcoholic potassium hydroxide solution only very slowly and with constant heating. The alkali consumption was equivalent to a molecular weight of 357 on the assumption that the product was monobasic. After recrystallization of the recovered acid it was found to melt at 82–83°. It was later discovered that the same product could be isolated from the freshly prepared unsaturated acid fraction by simply dissolving the acids in petroleum ether and allowing them to stand overnight at a temperature of about 10°. Although the substance could not be definitely characterized, it appeared to be an oxidation product derived from the unsaturated acids.

By a repetition of the process of cooling to low temperature and filtering the petroleum ether solution, the iodine number of the oleic acid was gradually raised from 80.6 to 85.9. Further purification by this process was not possible. Therefore a portion of the acid was distilled in a molecular still (13). The bulk of the acid distilled between 100–106°. The largest fraction distilled at 100°. It was water-clear at room temperature and had an iodine number of 91.1. Theory for oleic acid is 89.9. Oxidation by alkaline permanganate according to the method of Lapworth and Mottram (14) gave dihydroxystearic acid melting at 132–133°.

*Saturated Fatty Acids*—The solid fatty acids (16 gm.) derived from the lead salt-ether separation had an iodine number of 13.9. They were esterified with 5 per cent hydrochloric acid in absolute ethanol. The bulk of the solvent was distilled off and the residue

poured into water and extracted with ether. The ethereal solution was washed with dilute sodium carbonate solution and then with water. The ether was removed by evaporation and the residual mixed esters fractionally crystallized from ethyl acetate. Three main fractions were obtained as follows: Fraction I melting at 55.8–56.0°, Fraction II which melted just above room temperature, and Fraction III which was entirely liquid at room temperature.

*Palmitic and Stearic Acids*—The liquid esters (Fraction III) were saponified and the recovered acids converted into the corresponding *p*-phenylphenacyl derivative according to the directions of Drake and Bronitsky (15). The phenacyl derivative (7.0 gm.) melted at 89.5–89.8° and gave on analysis C 80.64, H 9.80. The phenacyl derivative appeared to be a mixture of palmitic and stearic acid esters but no marked separation could be effected by fractional crystallization from solvents. Therefore the acid was regenerated from a portion of the ester. It melted at 58.0–58.5° and had a molecular weight of 269.3, which correspond to the properties of an equimolar mixture of palmitic and stearic acids, molecular weight 270.2.

*Acids Higher Than C<sub>18</sub>*—The esters and the derived acids, representing Fractions I and II, were repeatedly fractionated by crystallization from solvents, which resulted finally in four main fractions. The topmost fraction melted at 84.0–84.5° and set to a crystalline solid at 84.0°. The x-ray photograph was poor and not measurable. The derived ethyl ester melted at 65.0° and set solid at 64.6–64.3°. The x-ray photograph<sup>3</sup> showed the presence of two different solid phases having spacings of 42.50 and 40.6 Å. The acid obtained on saponification of the ester melted at 82.0–82.2° and set to a crystalline solid at 80.5°. The mean molecular weight (440.2) by titration corresponds to a chain length of 29 carbon atoms (438.5). The data recorded here correspond to those given in the literature for montanic acid which has been shown by Francis, Piper, and Malkin (16) to consist of a mixture of C<sub>28</sub>, C<sub>30</sub>, and C<sub>32</sub> acids.

The acid representing Fraction II melted at 76.5° and set solid at 74.5–74.3°. The spacing of the acid was 62.20 Å. and that of

<sup>3</sup>All x-ray spacing photographs reported throughout this paper were made on pressed specimens with Cu *K* radiation with  $\beta$ -Al<sub>2</sub>O<sub>3</sub> as a reference.

the ethyl ester 39.5 Å. The ester melted at 60.7–61.0° and set solid at 59.8–59.5°. The molecular weight (385.0) by titration corresponds to an average chain length of 25 carbon atoms (382.4). The values recorded here correspond to those found in the literature (16) for cerotic acid which has been found to consist of a mixture of  $C_{24}$ ,  $C_{26}$ , and  $C_{28}$  acids.

The acid representing Fraction III melted at 69.0–70.0° and set to a crystalline solid at 68.5°. The corresponding ethyl ester melted at 59.0–59.3° and set to a crystalline solid at 58.8–58.4°. The x-ray spacing of the acid was 60.20 Å. and that of the ethyl ester 37.0 Å., corresponding to a mean chain length of 25 carbon atoms.

It may be concluded that the components of the saturated fatty acid fraction derived from grape pomace are principally palmitic and stearic acids together with the higher acids containing up to 32 carbon atoms. Two of the fractions correspond to montanic and cerotic acids which are, however, not pure substances but varying mixtures of higher saturated fatty acids.

#### *Unsaponifiable Matter*

The unsaponifiable fraction (54 gm.) was extracted with ether. A quantity (14 gm.) of bluish black oil remained insoluble. Further quantities of oil separated at various stages of treatment of the unsaponifiable fraction. The total oily material collected at all stages amounted to approximately 40 per cent of the unsaponifiable fraction. Since no crystalline product or derivative could be isolated from the oily fraction, it was discarded.

*Nonacosane,  $C_{29}H_{60}$ , and Hentriacontane,  $C_{31}H_{64}$* —A portion (10 gm.) of the unsaponifiable matter (iodine number 55.4) was treated with concentrated sulfuric acid at 120° for 20 hours. After cooling, the mixture was poured into a large volume of water and the solid which separated was filtered off. The washed and dried solid was extracted with petroleum ether and the petroleum ether solution filtered and evaporated. The residue was again treated with sulfuric acid and the process repeated as before until the addition of fresh acid to the recovered product no longer resulted in the formation of color. The final product was crystallized several times from a mixture of petroleum ether and acetone (1:1),

after which it melted at 64.9–65.2° and set to a crystalline solid at 64.3–64.0°. The x-ray spacing was  $40.20 \pm 0.30$  Å. Comparison of these values with those for pure synthetic hydrocarbons and their mixtures (17) indicates that the hydrocarbon derived from grape pomace consisted of approximately 70 per cent nonacosane and 30 per cent hentriacontane.

*Sitosterol*,  $C_{29}H_{50}O$ —A portion (8 gm.) of the unsaponifiable fraction was acetylated by refluxing with acetic anhydride. The recovered product was fractionally crystallized from ethanol and then from acetone. The two lowest melting fractions (59.0° and 63.0°) which amounted to 3.5 gm. gave no Liebermann-Burchard reaction. They appeared to consist principally of impure hydrocarbon. The next two fractions which melted at 82° and 90° gave strong sterol reactions. They were therefore combined (3.0 gm.) and saponified with sodium ethoxide. The recovered product was fractionally crystallized from acetone and then from absolute methanol. The melting point rose to 136.0–136.8° and was unchanged by further crystallization. The product was reacetylated and the acetyl derivative repeatedly crystallized. The melting point of 122.0–122.5° remained unchanged on further crystallization. After drying in a high vacuum at 80°, analysis gave C 81.52, H 11.60. Sitosteryl acetate,  $C_{31}H_{52}O_2$ , requires C 81.50, H 11.48. A portion of the acetyl derivative was saponified and the recovered sterol crystallized from ethanol from which it was obtained in the form of lustrous plates melting at 136.5–137°. Analysis gave C 84.13, H 12.02. Sitosterol requires C 83.98, H 12.16.

*Primary Alcohols*—In order to isolate the alcoholic constituents, a further quantity of unsaponifiable matter was prepared. In this preparation the wax constituents were first separated from the fatty materials by precipitation of the former from ether solution by the addition of acetone in accordance with the directions of Pollard, Chibnall, and Piper (18). The unsaponifiable matter prepared from the ether-acetone-insoluble fraction contained no oily material and gave no Liebermann-Burchard reaction. A portion was phthalated by refluxing for 18 hours with phthalic anhydride in pyridine solution. The reaction product was freed of pyridine and excess reagent, after which it was dissolved in ether and shaken with warm dilute sodium carbonate solution.

The supernatant ether solution was separated from the emulsified aqueous layer and washed repeatedly with water. The ether was removed and the residue taken up in ethanol in which it was very soluble. No separation occurred until the volume had been reduced one-half, when a product crystallized out which melted at 73.5–75.5°. Further concentration resulted in the separation of an additional quantity of material melting at 72–73°.

The aqueous emulsified layer remaining after separation of the ethereal portion was centrifuged and the precipitate washed with water. It was suspended in ether and shaken with dilute hydrochloric acid, which resulted in the complete solution of the solids by the ether. After washing with water the ether was removed by evaporation. The residue after crystallization from ethanol melted at 70.5–72.0° and appeared to be quite similar to the ether-soluble fractions previously obtained. The three fractions were therefore combined and saponified with sodium ethoxide. The recovered product was fractionally crystallized from methanol, acetone, and petroleum ether. Altogether eighteen fractions were obtained which were finally reduced to three main fractions.

The topmost fraction melted at 79.5–79.7°. Analysis gave C 81.62, H 14.41. Calculated for  $C_{27}H_{56}O$ , C 81.72, H 14.24. A portion of the product was acetylated and the acetyl derivative fractionally crystallized. Two acetates were obtained which melted at 61.2° and 60.5–60.7°. These results are in agreement with those recorded in the literature for so called ceryl alcohol (m.p. 79–80°) formerly presumed to have the formula  $C_{27}H_{56}O$ , but more recently (18) shown to consist of a mixture of primary alcohols containing only even numbers of carbon atoms, usually  $C_{26}H_{54}O$  and  $C_{28}H_{58}O$ .

The second alcohol fraction melted at 75.4–75.7°. The x-ray spacing value was  $71.20 \pm 0.40$  Å. Analysis gave C 81.56, H 14.27. Calculated for  $C_{28}H_{58}O$ , C 81.43, H 14.23. Fractionation of the derived acetates gave two fractions, the first of which melted at 63.2–63.5° and gave an x-ray spacing of  $79.60 \pm 0.30$  Å. The second acetate fraction melted at 58.2–58.5° and gave an x-ray spacing of  $75.80 \pm 0.30$  Å. These values correspond to an alcohol of mean molecular weight corresponding to  $C_{28}H_{58}O$ . Such alcohol mixtures have been isolated from various natural sources and assigned names like neoceryl alcohol. The latter has been

shown (19) to consist of a mixture corresponding to 40 per cent  $C_{24}$ , 40 per cent  $C_{26}$ , and 20 per cent  $C_{28}$  alcohols. The corresponding alcohol from grape pomace consists essentially of a mixture of  $C_{24}$  and  $C_{26}$  alcohols with only a small amount of  $C_{28}$  alcohol.

The third and final alcohol fraction melted at  $73.2^{\circ}$ . Analysis gave C 81.58, H 13.93. Calculated for  $C_{24}H_{50}O$ , C 81.26, H 14.22. The corresponding acetate melted at  $56.8-57.0^{\circ}$ . In the absence of data for known mixtures of pure alcohols of these compositions it is not possible to suggest the composition of this fraction, although extrapolated values indicate a mixture of  $C_{22}$ ,  $C_{24}$ , and  $C_{26}$  alcohols.

### *Ether Extract*

The ether-soluble residue, obtained by extracting grape pomace which had previously been extracted with petroleum ether as has been described in an earlier part of this paper, consisted of a greenish yellow resinous powder. The whole fraction (203 gm.) was boiled with 2 liters of water containing 40 gm. of sodium hydroxide. The insoluble portion was separated by filtration on a Buchner funnel, washed with hot water, and again boiled with more dilute sodium hydroxide solution. The insoluble portion was separated by filtration, washed with hot water, and dried. The recovered sodium salt weighed 109 gm., equivalent to 51.2 per cent of the original material.

*Oleanolic Acid*,  $HO \cdot C_{29}H_{46} \cdot COOH$ —The crude sodium salt was digested on the steam bath with ethanol containing a small amount of sodium hydroxide. The hot solution was filtered to remove insoluble gummy matter. The alcoholic filtrate was heated and stirred mechanically while an equal volume of boiling water was added. The crystalline salt which slowly separated as the solution became concentrated was filtered and the insoluble portion treated twice more by the same process. The residual salt was dissolved in hot ethanol and to the stirred solution a large volume of hot dilute hydrochloric acid was added. The precipitated acid after being filtered, washed, and dried weighed 64 gm. A further 30 gm. were obtained by reworking the collected filtrates. The total quantity of oleanolic acid recovered amounted to 46.3 per cent of the ether extract.

After recrystallization from ethanol solution the acid was ob-

tained in the form of colorless rods melting at 310–310.5°. Mr. George L. Keenan of the Food and Drug Administration, United States Department of Agriculture, reports the following optical properties for the acid: "In parallel polarized light (crossed nicols), the sign of elongation is  $\pm$  and the extinction parallel. Only a partial biaxial interference figure is observable in convergent polarized light (crossed nicols). The refractive indices as determined by the immersion method are  $n_\alpha = 1.520$  (shown crosswise on the rods, but not common);  $n_\beta = 1.533$  (shown lengthwise on rods, and most common of the indices);  $n_\gamma = 1.552$  (shown crosswise on rods, but not common);  $n_\gamma - n_\alpha = 0.032$ ; all  $\pm 0.003$ ."

When the product is dissolved in chloroform and treated with 5 drops of acetic anhydride and 1 drop of sulfuric acid (Liebermann-Burchard reagent), a transient pink color is developed which becomes pinkish violet and finally purple, showing a strong green fluorescence. After drying at 160° analysis gave C 78.89, H 10.65. Oleanolic acid,  $C_{30}H_{48}O_3$ , requires C 78.88, H 10.60.

*Acetylmethyloleanate*,  $CH_3CO \cdot O \cdot C_{29}H_{46} \cdot COCH_3$ —A portion of the purified oleanolic acid was methylated with methyl iodide in the usual manner. The methyl ester, after crystallization from 70 per cent ethanol, melted at 200–201°. The product was acetylated by refluxing with acetic anhydride. After crystallization from dilute ethanol the acetylated ester melted at 221–222°. After drying at 125°, analysis gave C 77.16, H 10.30. Acetylmethyloleanate,  $C_{33}H_{52}O_4$ , requires C 77.28, H 10.23.

*Diacetyloleanolic Acid*,  $CH_3CO \cdot O \cdot C_{29}H_{46}COO \cdot COCH_3$ —A portion (15 gm.) of oleanolic acid was refluxed with acetic anhydride and allowed to cool, whereupon the acetylated derivative separated from the acetic anhydride solution. The product (14 gm.) was recrystallized from acetic anhydride and then dried *in vacuo* over powdered potassium hydroxide. Placed in a bath at 250° the compound melted with evolution of gas, resolidified, and melted again at 320–323°. One specimen placed in the bath at about 150° melted at 190°, resolidified, and melted at the higher temperature. Another specimen which was heated more slowly did not melt but underwent a marked change in crystalline structure and volume at 210–230° and finally melted at 320°. Analysis gave C 75.68, H 9.66. Diacetyloleanolic acid,  $C_{34}H_{52}O_5$ , requires C 75.50, H 9.70.



*Monoacetyloleanolic Acid*,  $\text{CH}_3\text{CO}\cdot\text{O}\cdot\text{C}_{29}\text{H}_{46}\text{COOH}$ —The diacetyl derivative was converted into the monoacetyl by refluxing it a short time with 70 per cent ethanol. The product after crystallization from ethanol melted at  $264.5\text{--}265.5^\circ$ . The acetyl derivatives differ markedly from oleanolic acid in their behavior toward the Liebermann-Burchard reagent. In contrast to the play of colors given by oleanolic acid the acetyl derivatives give an intense cherry-red coloration under the same conditions. Analysis of the monoacetyl derivative gave C 76.98, H 10.02. Monoacetyloleanolic acid,  $\text{C}_{32}\text{H}_{50}\text{O}_4$ , requires C 77.05, H 10.11.

$\delta$ -*Ketoacetyloleanolic- $\gamma$ -Lactone*,  $\text{C}_{32}\text{H}_{48}\text{O}_5$ ,—Kitasato and Sone (20) oxidized monoacetyloleanolic acid with chromic acid in glacial acetic acid and obtained as the principal product of oxidation  $\delta$ -ketoacetyloleanolic- $\gamma$ -lactone, melting at  $277\text{--}280^\circ$ . It was thought that oxidation of the diacetyl derivative under the same conditions might give the corresponding acid as the principal product, since lactonization might be expected to be inhibited by blocking of the carboxyl group in the case of the mixed anhydride. Oxidation of the mixed anhydride (diacetyl derivative) does lead to somewhat different products, but nevertheless about one-third of the reaction mixture consists of the lactone obtained by Kitasato and Sone.

The diacetyl derivative (14 gm.) was suspended in 600 ml. of glacial acetic acid containing 10 ml. of acetic anhydride. 125 ml. of glacial acetic acid containing 12 gm. of chromic anhydride were added slowly over a period of 2 hours, during which time the reaction mixture was shaken mechanically. The shaking was continued for 7 hours, after which the mixture was allowed to stand overnight. Methyl alcohol was then added slowly with cooling to destroy the excess of chromic acid. The solvent was removed by distillation *in vacuo* and water added to the residue. The addition of ether resulted in the formation of an emulsion; consequently alkali was added until the mixture separated into two layers. The upper, ethereal layer was clear and colorless and the lower aqueous layer greenish and contained considerable suspended material. The two layers were separated and examined.

The washed ethereal layer was concentrated and the residue taken up in hot absolute methanol. On standing for some time the solution deposited a mixture of two very different types of

crystalline material; namely, long thin blades and a smaller amount of massive cubes. The mixed crystalline material (3 gm.) was separated by filtration and the solid extracted with acetone in which the blades were soluble and the cubes only slightly so. By repetition of the process of crystallization from methanol and extraction of the crystalline material with acetone a product was obtained which was entirely homogeneous and consisted of slender blades about 1 cm. in length. It melted at 279–280°. After drying in a high vacuum, analysis gave C 74.85, H 9.53.  $\delta$ -Ketoacetyloleanolic- $\gamma$ -lactone,  $C_{33}H_{48}O_6$ , requires C 74.94, H 9.44.

#### SUMMARY

Grape pomace derived from Concord grapes (*Vitis labrusca*) has been investigated with respect to the constituents present in the petroleum ether and ether extracts. The total ether-soluble extract amounts to 7.4 per cent of the air-dried pomace. Approximately one-half (54 per cent) of the total ether-soluble constituents was extractable by petroleum ether.

After saponification of the petroleum ether extract the following substances were identified: *glycerol*,  $C_{18}H_{32}O_2$ , and *oleic*,  $C_{18}H_{34}O_2$ , acids; fractions representing mixtures consisting principally of *palmitic*,  $C_{16}H_{32}O_2$ , and *stearic*,  $C_{18}H_{36}O_2$ , acids and higher saturated fatty acids of the series  $C_{20}$  to  $C_{32}$ ; the hydrocarbons, *nonacosane*,  $C_{29}H_{60}$ , and *hentriacontane*,  $C_{31}H_{64}$ ; *sitosterol*,  $C_{29}H_{50}O$ , and fractions representing mixtures of primary alcohols of the series  $C_{22}$  to  $C_{28}$ . The fraction obtained by extraction of the grape pomace with ether after previous extraction with petroleum ether was found to consist principally of *oleanolic acid*,  $C_{30}H_{48}O_3$ , together with unidentified resinous substances.

In view of the interest which is being displayed by many workers in the structural chemistry of ursolic and oleanolic acids, we wish to point out the importance of apple and grape pomace, respectively, as inexpensive and readily available sources of these two interesting sapogenins.

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# THE CATABOLISM OF THE PURINE NUCLEOTIDES

## I. THE RELATION TO GLYCOLYSIS IN THE BLOOD OF THE RABBIT\*

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Since the first isolation of adenylic acid from hog blood by Hoffman (1), and from muscle by Embden and Zimmermann (2), many recent investigations have shown that other adenine-containing nucleotides, such as adenylypyrophosphoric acid (3-5), codehydrogenase I (6), and codehydrogenase II (7), are of widespread occurrence in animal tissues. The literature that deals with the investigations concerning the chemical and physiological properties of the nucleotides that are mentioned above is very extensive and need not be reported here.

For the purpose of the discussion that is to follow, it will suffice to mention that one of the properties which is attributed to the adenine nucleotides is participation in the enzymic reactions that are involved in the catabolism of carbohydrates. It is generally accepted that adenylypyrophosphoric acid, which constitutes the greater part of the adenine nucleotides present in tissues, functions in certain of the reactions of glycolysis that involve the transfer of phosphate from one organic compound to another.

In addition to their function as part of certain enzymic systems, the adenine nucleotides are themselves subject to the action of the enzymes of certain tissues. An adenylypyrophosphatase that catalyzes the dephosphorylation of adenylypyrophosphoric acid to adenylic acid has been described by Jacobsen (8). Schmidt (9) has studied the occurrence and distribution of adenylic acid deaminase which catalyzes the deamination of adenylic acid to inosinic acid. An adenosine deaminase has also been described by

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Schmidt. The catalytic action of nucleotidase, as well as nucleosidase, has been studied by Levene and various other workers (10). An enzyme that is capable of catalyzing the hydrolysis of the glucosidic linkage of a nucleotide has been described recently by Ishikawa and Komita (11). In other words, the decomposition of the adenine nucleotides may involve any one or all of the following reactions: dephosphorylation, deamination, hydrolysis, or oxidation.

Certain phases of the reactions that are mentioned briefly in the foregoing paragraphs have been the subject of numerous investigations. However, aside from the many works concerning the formation of ammonia in tissues, and those pertaining to the stability of adenylypyrophosphoric acid during glycolysis, to our knowledge there have been no investigations that involved the study of the pathway of the catabolism of the adenine nucleotides and its relation to glycolysis. The experiments that are to be discussed were conducted in an endeavor to fill in the omission.

#### EXPERIMENTAL

About 40 cc. of blood were obtained by cardiac puncture from each of four adult, unanesthetized rabbits. The samples of blood were pooled and defibrinated. The defibrinated blood was placed in an incubator at 37° under aerobic conditions. Initial samples for analyses were taken one-half hour after the blood was drawn from the first of the four rabbits. Thereafter, in certain of the experiments, samples were taken for analyses at intervals of 1 to 2 hours for the first 8 to 9 hours of a period. In other experiments, the first sample after the initial sample was taken at the end of 6 to 8 hours, with samples at 1 to 2 hour intervals thereafter. In this manner, data were collected for a period of 15 hours.

The following analytical methods were used: glucose, the colorimetric method of Benedict (12); lactic acid, the method of Friedemann, Cotonio, and Shaffer (13), with the refinements in apparatus and technique as described by West (14) and Friedemann and Graeser (15). Inorganic phosphate was determined by the method of Fiske and Subbarow, as modified by Lohmann and Jendrassik (16). The method of Lohmann and Jendrassik includes the phosphate of phosphocreatine as well as inorganic phosphate. The pyro fraction phosphate was determined by the

method of Lohmann (17). The phosphate that is estimated by this method is derived almost exclusively from two-thirds of the phosphate of adenylypyrophosphoric acid. Uric acid was determined by the method of Benedict and Behre (18). The nucleotide nitrogen and the sum of the nucleoside nitrogen and free purine nitrogen were determined by the method of Kerr and Blish (19). When the method was published (1932), the presence of codehydrogenase I and codehydrogenase II in mammalian tissues was not known. Accordingly, the method was published without reference to the specific nucleotides that are estimated. It is now clear that inosinic acid, adenylic acid, adenylypolyphosphoric acids, codehydrogenase I (20), and codehydrogenase II (21) are included in the nucleotide fraction. The nucleoside plus free purine fraction includes all purine nucleosides and free purines.

All analyses were carried out in duplicate. Results that did not check within the experimental accuracy of the method were discarded. The average value of check results was used.

The data for three out of a total of nine experiments are presented graphically in Figs. 1 to 3. In all of the experiments the initial values for the content of lactic acid in blood (74 to 136 mg. per cent) are high. Blood that is obtained by cardiac puncture from unanesthetized rabbits almost always has a high concentration of lactic acid (22). The rate of formation of lactic acid ranged from 8 to 12 mg. per cent per hour.

The initial values for the glucose content of the blood ranged from 89 to 109 mg. per cent. 80 to 100 per cent of the glucose that disappeared from the blood was accounted for as lactic acid. As would be expected, the duration of glycolysis varied with the initial concentration of glucose and the rate of glycolysis. In general, the formation of lactic acid continued over a period of from 6 to 9 hours.

The initial values for the content of inorganic phosphate, in terms of P, ranged from 3.2 to 3.8 mg. per cent. The initial values for the content of pyro fraction phosphate, in terms of P, ranged from 3.9 to 5.0 mg. per cent. The initial values for the content of nucleotide nitrogen ranged from 6.8 to 7.2 mg. per cent, and the initial values for the content of nucleoside nitrogen plus free purine nitrogen ranged from 1.0 to 1.5 mg. per cent. Since the pyro fraction phosphate is equal to two-thirds of the phosphate content

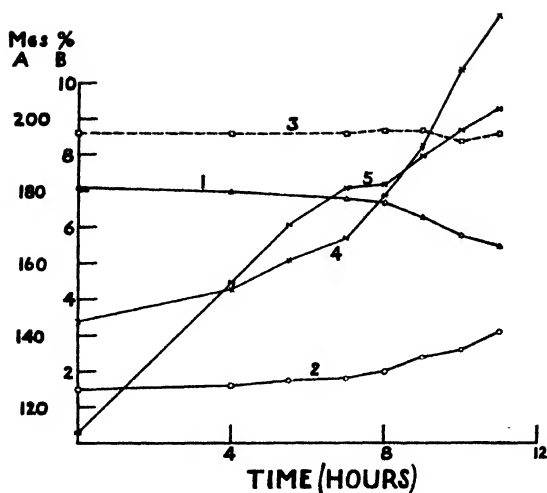


Fig. 1. The decomposition of the purine nucleotides and the glycolysis of the whole blood of the rabbit under aerobic conditions. Curve 1, Scale B, nucleotide nitrogen; Curve 2, Scale B, nucleoside nitrogen plus free purine nitrogen; Curve 3, Scale B, total acid-soluble purine nitrogen; Curve 4, Scale B, inorganic phosphate P; Curve 5, Scale A, lactic acid.

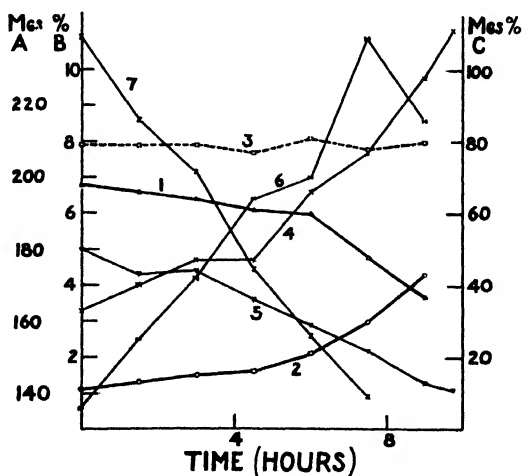


Fig. 2. The decomposition of the purine nucleotides and the glycolysis of the whole blood of the rabbit under aerobic conditions. Curve 1, Scale B, nucleotide nitrogen; Curve 2, Scale B, nucleoside nitrogen plus free purine nitrogen; Curve 3, Scale B, total acid-soluble purine nitrogen; Curve 4, Scale B, inorganic phosphate P; Curve 5, Scale B, pyro fraction P; Curve 6, Scale A, lactic acid; Curve 7, Scale C, glucose.

of adenylypyrophosphoric acid, the initial value for the adenylypyrophosphoric acid nitrogen content may be obtained by multiplying the value for the pyro fraction phosphate content by 1.13. When such calculations are carried out, adenylypyrophosphoric acid nitrogen is found to account for 72 to 83 per cent of the total content of nucleotide nitrogen. The remainder of the nucleotide nitrogen consists of codehydrogenase I, codehydrogenase II, and possibly adenylic acid.

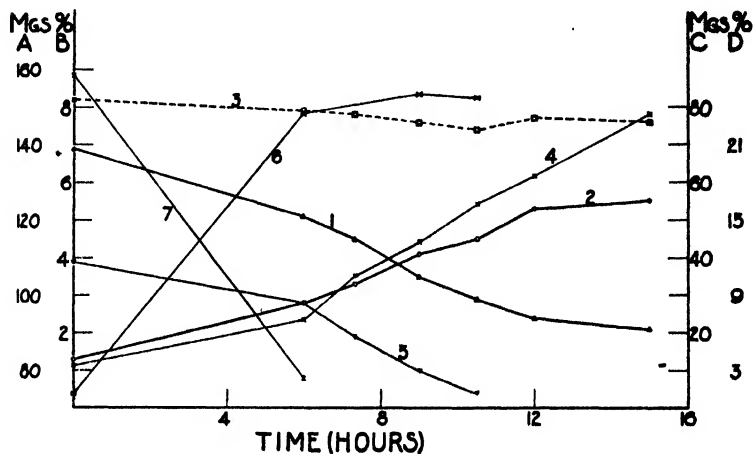


FIG. 3. The decomposition of the purine nucleotides and the glycolysis of the whole blood of the rabbit under aerobic conditions. Curve 1, Scale B, nucleotide nitrogen; Curve 2, Scale B, nucleoside nitrogen plus free purine nitrogen; Curve 3, Scale B, total acid-soluble purine nitrogen; Curve 4, Scale D, inorganic phosphate P; Curve 5, Scale B, pyro fraction P; Curve 6, Scale A, lactic acid; Curve 7, Scale C, glucose.

The data for the *period of glycolysis* (Figs. 1 and 2) show the following. (a) The total acid-soluble purine nitrogen content remains constant. The small fluctuations that are observed are within the experimental error. (b) The nucleotide nitrogen content remains relatively constant. Any changes that are noted are in the nature of a slow rate of decrease. (c) The nucleoside nitrogen plus free purine nitrogen fraction undergoes little or no change. Any changes that are noted are in the nature of slight increases which exactly compensate for any small decreases in the concentration of nucleotide nitrogen as noted in (b). (d) The



pyro fraction phosphate content decreases slowly. (e) The inorganic phosphate content increases slowly.

After the *cessation of glycolysis* (Fig. 3, data from the 6th to 15th hours), the data show the following. (a) The total acid-soluble purine nitrogen content remains constant. (b) The nucleotide nitrogen content decreases rapidly. (c) The nucleoside nitrogen plus free purine nitrogen fraction increases rapidly. The increase in concentration in this fraction is equal to the decrease in the concentration of nucleotide nitrogen as noted in (b). (d) The concentration of pyro fraction phosphate decreases to zero. (e) The inorganic phosphate content increases rapidly.

The data for the uric acid content are not presented graphically. The values remained practically constant at 0.5 mg. per cent for the duration of all the experiments.

#### DISCUSSION

The investigations of Rona and Iwasaki (23) and of Engelhardt and coworkers (24-26) have demonstrated that the concentration of inorganic phosphate in samples of shed blood is dependent upon the rate of glycolysis. According to the latter workers, a constant synthesis and hydrolysis of organic phosphate esters accompany glycolysis. The net result of the synthesis and hydrolysis is such that during the period of rapid glycolysis there is little change in the concentration of inorganic phosphate. The hydrolysis of phosphate esters is observed only in the absence of glycolysis.

It is known now (27) that the phosphate interchange that takes place in glycolysis as observed in blood is due mainly to the ability of adenylypyrophosphoric acid to donate phosphate to hexose-monophosphate and possibly also to hexose. Thus, hexosediphosphate and adenylic acid (or possibly adenyldiphosphoric acid) are formed. On the other hand, adenylic acid or adenyldiphosphoric acid each may act as phosphate acceptor. In this case the phosphate is donated by phosphopyruvic acid. Adenylypyrophosphoric acid and pyruvic acid are formed as a result of the exchange. The formation of adenylypyrophosphoric acid from adenylic acid and inorganic phosphate is coupled with the oxidation and reduction reactions that take place between pyruvic acid and the triose phosphates that are in equilibrium with hexosediphosphate.

The end-effects of the various phosphorylation reactions are demonstrated very well by the data reported here. During the period of rapid glycolysis, the concentration of inorganic phosphate remains relatively constant. The concentration of pyro fraction phosphate decreases very slowly. When glycolysis ceases (owing to the depletion of glucose), there is observed (a) a rapid increase in the concentration of inorganic phosphate, and (b) a rapid decrease in the concentration of pyro fraction phosphate.

The catabolic phase of the nucleotide metabolism begins at the termination of glycolysis. During glycolysis, adenylypyrophosphoric acid maintains its *status quo*. At the cessation of glycolysis, the reactions which normally rephosphorylate adenylic acid no longer take place. In consequence thereof, adenylic acid accumulates. The adenylic acid is then subject to the catalytic action of nucleotidase. Nucleotidase action is demonstrated in the experiments reported here by the rapid decrease in the concentration of nucleotide nitrogen that is observed to occur at the termination of glycolysis.

Since there are no demonstrable amounts of nucleosidase present in rabbit blood (28), it is quite probable that the catabolism of the purine nucleotides within the red corpuscles ends with the formation of adenosine. In agreement with the experiments of Engelhardt (29), the formation of uric acid was observed not to occur.

Mozolowski (30) has ascribed a part of the ammonia formation that takes place in blood to the deamination of purine nucleotides. The experiments of Mozolowski were conducted over longer periods than those that are described in this paper. The results reported here do not show any decrease in acid-soluble purine nitrogen other than that which is due to inherent experimental errors. If deamination of adenylic acid were to occur (in the intact red corpuscle), the value for the acid-soluble purine nitrogen should decrease by one-fifth. Thus, it may be that the results of Mozolowski are due to hemolysis of the red corpuscles, since Heller and Klisiecki (31) have shown that, in the case of sheep blood, deamination of purine nucleotides occurs only after hemolysis. Further, it is not possible for any decrease in acid-soluble purine nitrogen to be masked by a neo-formation of acid-soluble purine that may be derived from the hydrolysis of the nucleic

acid which is present in the white cells, since Jono (32) has found that the nuclease activity of rabbit blood is nil. The deamination of purine nucleotides that was noted by Mozolowski must take place solely in the plasma.

The foregoing data show that the rate of catabolism of the purine nucleotides in blood depends upon the rate of glycolysis. It is to be expected that the conditions which are represented on a magnified scale in the experiments (through the depletion of the glucose content of the blood) occur continuously on a smaller scale during the metabolism *in vivo*. For example, during normal glycolysis (27) small amounts of adenylic acid must escape resynthesis to adenylypyrophosphoric acid, and thus fall into the catabolic phase. In this manner, adenosine is formed. The further catabolism of the nucleoside possibly depends upon its diffusion from the red corpuscle.

#### SUMMARY

1. The decomposition of the purine nucleotides and the glycolysis of the defibrinated whole blood of the rabbit have been studied.

2. A relationship between glycolysis and the stability of the purine nucleotides has been found. During glycolysis, the purine nucleotides maintain their *status quo*. At the termination of glycolysis, the purine nucleotides are decomposed. The decomposition of the purine nucleotides proceeds via dephosphorylation. There is no loss of purine nitrogen either through deamination or oxidation. The end-product of the catabolism of the purine nucleotides, within the red blood corpuscle, possibly is adenosine.

3. The relationship between the catabolism of the purine nucleotides and glycolysis has been discussed.

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## **A VOLUMETRIC BENZIDINE METHOD FOR THE DETERMINATION OF INORGANIC AND ETHEREAL SULFATE IN SERUM**

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Several years ago, Hubbard (1, 2) demonstrated that extremely small quantities of sulfate present in trichloroacetic acid filtrates of human serum can be isolated by precipitation as benzidine sulfate. Serum was deproteinized with an equal volume of 20 per cent trichloroacetic acid, and 2 cc. of the protein-free filtrate were added to 5 cc. of a 1 per cent solution of benzidine in acetone. The precipitate of benzidine sulfate that formed was separated and washed with acetone by means of the centrifuge; it then was treated with ferric chloride and hydrogen peroxide which reacted with the benzidine of the precipitate and produced a yellow color.

Several modifications of this procedure have been proposed, some of which have been concerned with the conditions of precipitation of the benzidine sulfate (3), others with attempts to develop a better color than the rather unsatisfactory yellow (4) or to replace the colorimetric feature of the method with some form of titration. Cope (5) subjected the benzidine sulfate to direct alkalimetric titration with the aid of a Rehberg burette; Power and Wakefield (6) oxidized the precipitate with sulfuric acid and dichromate and estimated the excess dichromate iodometrically.

Obviously, the use of any of these modified procedures as well as the original method itself is based on the assumption that the precipitate of benzidine sulfate is reasonably pure. According to our experience (7, 8) this assumption is not justified, as comparison of the oxidimetric and alkalimetric titration methods indicates

that considerable quantities of oxidizable impurities may be associated with benzidine sulfate when it is separated according to the conditions outlined by Hubbard. These impurities cause the results of the oxidimetric method to be too high, and, in addition, are not without adverse effect on the alkalimetric titration. We have found, however, that a compensating error occurs; namely, that the recovery of quantities of sulfate comparable to those present in normal human serum is only 75 to 90 per cent complete. This may account, we believe, for the fact that the oxidimetric method has, in our hands as well as in the hands of others, supplied rather reasonable data as to the amount of inorganic sulfate in human serum (9-11).

In subsequent investigations we have found that the use of 95 per cent ethyl alcohol (12) instead of acetone in the precipitation of benzidine sulfate from trichloroacetic acid filtrates eliminates many of the difficulties and inconsistencies that have been encountered. The precipitate has the characteristic white silky appearance and is much less contaminated with impurities than the corresponding precipitate from an acetone medium. For the estimation of the precipitate we prefer alkalimetric titration to colorimetric or oxidimetric methods. The alkalimetric titration may be accomplished in various ways, as with 0.02 N solution of sodium hydroxide and the Rehberg microburette according to Cope (5), or with more dilute alkali and some form of the ordinary microburette usually at hand in the laboratory. A procedure of the latter type, in which phenol red is used as indicator, will be described.

#### *Reagents—*

Trichloroacetic acid (redistilled<sup>1</sup>). A solution containing 20 gm. in 100 cc. is prepared as follows: The acid is melted in a water bath at about 65°, 25 cc. of the melted acid are transferred to

<sup>1</sup> Trichloroacetic acid was purified by distillation *in vacuo*, in a 2000 cc. Pyrex distilling apparatus (No. 1370) connected by means of a rubber stopper to a suction flask as receiver. Hot water was used in the condenser. Considerable bumping occurred, in spite of the use of various antibumping devices; nevertheless, in the distillation of about a kilo of the acid, to which 1.0 gm. of benzidine was added to combine with the sulfate, a colorless and practically sulfate-free product was obtained.

distilled water by means of a previously warmed pipette, and the volume is made up to 200 cc.

Ethyl alcohol, 95 per cent. Ordinary alcohol was redistilled; this gave the same results as aldehyde-free preparations.

Benzidine in alcohol. 1 gm. of benzidine is dissolved in alcohol and the solution made to a volume of 100 cc. Solutions of purified<sup>2</sup> benzidine will keep from 1 to 2 weeks in a brown, glass-stoppered bottle.

Sodium hydroxide. A 0.01 to 0.001 N solution is used, depending on the size and graduation of the burette to be used. For routine determinations the use of 0.002 N solution in a 5 cc. burette graduated to 0.01 cc. and provided with a Shohl (14) needle tip is convenient; one filling of the burette serves for several titrations. The solution of the alkali is prepared essentially free of silicate and carbonate according to the method of Fiske and Logan (15). The solution is preserved in a bottle coated with a purified grade of paraffin and provided with a soda lime guard tube. The factor of a 0.001 or 0.002 N solution does not change materially for several months. The burette should be filled without exposure of the solution to air. Fiske and Logan described a suitable procedure, and various other ways will suggest themselves. Since filling through the tip introduces traces of grease from the stop-cock, which necessitates more frequent cleaning, we prefer to fill through the top. A soda lime guard tube is then attached.

Phenol red. A 0.01 per cent aqueous solution of phenol red is used. This solution should be clear and free of suspended particles.

\* For the purification of benzidine, the method of Bing (13) was modified as follows: 20 gm. of benzidine were dissolved in 150 cc. of 70 per cent alcohol (7 volumes of 95 per cent alcohol plus 3 volumes of water) at about 70°, allowed to crystallize in the refrigerator, filtered with suction, and washed with a little cold 50 per cent alcohol. The crystals were redissolved in about 135 cc. of 70 per cent alcohol, 1 gm. of decolorizing carbon (carboraffin) was added, and the mixture heated an additional 1 or 2 minutes, then filtered immediately on a hot water funnel through a paper which previously had been washed with some of the warm alcohol; finally, the flask and filter were washed with about 30 cc. of warm 50 per cent alcohol. The filtrate was cooled in the refrigerator, and the benzidine filtered with suction, and dried in a dark place. The air-dried substance contains water and traces of carbon which should be removed by recrystallization from dry benzene. The crystals were preserved in a bottle of brown glass.



*Method*

*Deproteinization*—Add 1 volume of 20 per cent trichloroacetic acid to 1 volume of serum (2 cc.) in a centrifuge tube, mix with a glass rod, carefully breaking up the curdy precipitate, and allow the mixture to stand 15 to 30 minutes, stirring thoroughly once or twice again. Close the tube with a rubber cap, centrifuge, and pour the supernatant fluid through a small sulfate-free filter paper. Filtrates made in this manner contain substantially the same amounts of sulfate as those from mixtures which are made to a definite mark in a volumetric flask before filtration. If the quantity of sulfate in the serum is greater than normal, remove proteins at a dilution of 1:5 or 1:10 by using for 1 volume of serum 4 volumes of 11.5 per cent or 9 volumes of 10 per cent trichloroacetic acid, respectively. The acidity of these various filtrates will correspond to about 8.5 per cent trichloroacetic acid for serum which contains a normal amount of protein. Variation of acidity from 7 to 10 per cent is without material influence on the result.

*Precipitation of Benzidine Sulfate*—Select a Pyrex centrifuge tube that is not scratched or etched on the inside and that is of such size and shape that the rounded end of a 3.0 to 3.5 mm. glass rod will make approximate contact with the inside surface at the tip. To 2 cc. of filtrate in such a tube add 5 cc. of benzidine reagent, stir the mixture with a glass rod, withdraw the rod, rinse it with a few drops of alcohol, close the tube with a rubber cap, and place it in the refrigerator. After an hour or more, centrifuge the tube for about 10 minutes at 2500 to 3000 revolutions per minute (the radius of the centrifuge head used was 14 cm.), decant the supernatant fluid carefully, allow the last drop or two to fall onto a clean towel, then invert the tube over the spot so moistened. Allow to drain about 5 minutes, wipe the mouth of the tube with a clean towel, add 5 to 6 cc. of alcohol, carefully rinse the sides from the top downward, pulverize the precipitate of benzidine sulfate with a moderately pointed Pyrex rod, remove the rod, rinse with a little alcohol, mix the precipitate and wash fluid by rolling the tube between the palms of the hands, add 5 to 6 cc. more of alcohol, again rinse the sides, roll the tube between the hands again, cap, and centrifuge the tube. Decant and drain over a moistened towel as before.

*Titration of Benzidine Sulfate*—Add 2.0 to 2.5 cc. of hot distilled water, rinse the walls of the tube, add 0.1 cc. of phenol red and a slender Pyrex rod, and place the tube in a beaker of water boiling over an electric plate. After a few minutes the precipitate will usually dissolve completely, especially if broken up by means of the rod. Remove the tube from the water bath, introduce a fine Pyrex jet for the delivery of carbon dioxide-free air,<sup>3</sup> and titrate with a 0.002 N solution of sodium hydroxide until the yellow of the indicator undergoes the first easily recognizable change of color, but avoid the production of much pink. Place the tube back in the water bath for  $\frac{1}{2}$  to 1 minute to insure solution of any undissolved benzidine sulfate and continue the titration if the yellow color returns. Duplicate determinations will usually agree to within 0.01 cc. or less. Subtract from the reading a blank value obtained by titrating indicator and water in a manner similar to the titration of benzidine sulfate. We have found this correction to be around 0.008 and 0.012 cc. at volumes of 2.5 and 5.0 cc. respectively. Likewise standardize the alkali by titrating say 2 cc. of 0.004 N acid as in an unknown titration, subtracting the value of the indicator-water blank before calculating the factor. When the amount of benzidine sulfate is large, the bulk of it may be dissolved and titrated without the introduction of the air stream; the tube then is replaced in boiling water to dissolve the remaining sulfate, the jet for delivery of carbon dioxide-free air is introduced, and the titration is completed. Calculation: If the benzidine sulfate from filtrate equivalent to 1 cc. of serum is titrated with 0.002 N solution of alkali, the amount of solution in cc. required minus the value of the indicator-water blank is multiplied by 3.2. This gives the inorganic sulfate in terms of mg. of S per 100 cc. of serum. If it is desired to express the result in terms of sulfate ( $\text{SO}_4$ ), the value so obtained is multiplied by 3.

*Determination of Total Sulfate*—Transfer 5 cc. of serum filtrate to a small Pyrex beaker, add 5 cc. of a 0.2 N solution of hydrochloric acid, cover the beaker with a watch-glass, and heat on the steam bath for 2 to 3 hours to decompose most of the trichloroacetic

<sup>3</sup> We have encountered samples of tubing which slowly liberate volatile bases in spite of careful cleaning with alkali, or subsequent soaking in dilute acid. Such tubing must not be used for the delivery of carbon dioxide-free air.

acid. Remove the cover and evaporate the contents of the beaker to dryness; finally, suspend the beaker in the steam bath by means of one of the supporting rings, and heat further for 3 to 4 minutes to expel traces of hydrochloric acid. After cooling, dissolve the residue in 5 cc. of 8.5 per cent trichloroacetic acid, filter or centrifuge if the solution is not clear, then proceed with the determination of sulfate. Experiments in which 5 cc. of filtrate were heated 20 to 30 minutes with 1 cc. of 6 N hydrochloric acid and then evaporated to dryness gave the same results as those in which the longer heating period was used.

*Cleaning of Glassware*—If the centrifuge tubes are soaked in sulfuric acid-dichromate solution, extreme care is required to remove the last trace of it. Rinse the tubes three to four times with tap water and two or three times with distilled water, then heat them an hour or more with hot distilled water; finally, again rinse the tubes four or five times with distilled water. Certain soft glass centrifuge tubes that have rolled rims sometimes retain solution under the rims. This later might constitute a source of contamination if such tubes were used in the procedure for protein precipitation.

#### EXPERIMENTAL

*Recovery of Sulfate from Inorganic Solutions*—Under the conditions outlined the recovery of sulfate from known solutions was slightly low by an amount corresponding, on the average, to 0.065 mg. of S per 100 cc. of serum; or, in other words, the recovery of 0.010 mg. of S was about 93.5 per cent complete; that of 0.0333 mg. was 98 per cent complete, and so forth. The recovery was not affected by the presence of inorganic phosphate, even in an amount 2 to 4 times that normally present, or by the presence of sodium chloride, or by the simultaneous presence of these and the various other inorganic constituents of serum. When the sulfate was precipitated with benzidine in acetone, however, and the precipitate washed with acetone, the recovery of sulfate from solutions that contained the added inorganic constituents was definitely depressed.

*Comparison of Volumetric and Gravimetric Determinations*—Microgravimetric determinations were conducted essentially as described by Pregl (16). The recovery of sulfate from known

solutions containing trichloroacetic acid and salts, as in a filtrate of normal serum, varied from 92.6 to 99 per cent (five experiments), and the average recovery was 95.8 per cent. The barium sulfate precipitates weighed 1.0 to 1.5 mg. The results of similar determinations on serum filtrates are compared with the values obtained by the volumetric method in Table I. In Experiments 1 to 6, representing analyses of normal serum, the values obtained by the benzidine method were 0.041 to 0.094 mg. of S per 100 cc. higher than those obtained by the gravimetric method. The mean difference, 0.075 mg. of S per 100 cc., possibly should be subtracted, as a correction, from the values obtained by the benzidine method.

TABLE I

*Comparison of Volumetric Benzidine and Microgravimetric Methods for Determination of Inorganic Sulfate in Serum*

Experiment No.	S in 100 cc.		Difference
	Gravimetric method	Volumetric method	
	mg.	mg.	per cent
1	0.829	0.870	+5.0
2	0.933	1.023	+9.6
3	1.037	1.087	+4.8
4	1.113	1.207	+8.4
5	1.150	1.230	+7.0
6	1.400	1.493	+6.6
7	3.773	3.893	+3.2
8 (Dog)	3.167	3.270	+3.3

The average deficiency of recovery of sulfate from known solutions by the benzidine method is of the same order of magnitude, however; consequently the use of such a correction would appear to be unnecessary. In Experiments 7 and 8 the differences between the results by the volumetric and gravimetric methods were, on the relative basis, much less than those encountered in the analysis of normal serum.

*Comparison of Alkalimetric Titration and Dichromate Oxidation of Benzidine Sulfate Precipitated from Alcohol and Acetone, Respectively*—The results of determinations in which the benzidine sulfate precipitates from alcohol were oxidized with dichromate agreed remarkably well with those obtained by alkalimetric titra-

tion of similar precipitates (Table II). With the precipitates obtained from acetone, however, the results obtained by the oxidation procedure were considerably higher than those obtained by the alkalimetric method and thus showed that such precipitates contained considerable quantities of oxidizable impurities. At the same time the alkalimetric titration of precipitates obtained from acetone gave lower results than the titration of precipitates from alcohol. This effect is due, in part at least, to the impurities present in the acetone precipitates. Evidence in support of this

TABLE II

*Alkalimetric Titration and Dichromate Oxidation of Benzidine Precipitates Obtained with Alcohol and Acetone*

The data are given in terms of mg. of S per 100 cc. of serum.

Serum No.	Alcohol precipitation		Acetone precipitation	
	Titration	Oxidation	Titration	Oxidation
1	1.11	1.13	0.97	1.27
2	1.06	1.09	0.94	1.23
3	1.14	1.16	1.03	1.37
4	1.49	1.51	1.46	
5	1.22	1.29	1.12	1.42
6	1.21	1.23		
7	0.96	1.00		
8	1.19		1.07	
9	1.28		1.22	
10	1.17		1.06	
11	1.14		1.05	
12	1.10		1.04	

view was obtained from experiments in which the benzidine sulfate precipitates were ashed with nitric acid, and from a study of serum filtrates from which the sulfate had been removed by means of barium chloride.

*Recovery of Sulfate Added to Serum*—A solution of potassium sulfate was added to serum in the proportion of 1 cc. to 20 cc. of serum; to another part of the serum, water was added in the same proportion. The mixtures were allowed to stand 20 to 40 minutes, after which the proteins were removed by precipitation with an equal volume of 20 per cent trichloroacetic acid. Analyses of 2 cc. portions of the filtrates were conducted in the usual manner;

in addition, analyses of 1 cc. portions (plus 1 cc. of 8.7 per cent trichloroacetic acid) of the filtrates obtained from the mixtures containing added sulfate were made. The average amount of sulfate recovered in these two series (Experiments 1 to 9, Table III) was found to be 103.6 and 96.8 per cent, respectively, of the quantity of sulfate added. This relationship seems reasonable, in the light of the results obtained with known solutions and the gravimetric comparisons. The percentage of sulfate recovered was rather satisfactory, particularly so when it is considered that the errors in the determinations all fall on the calculated values for sulfate recovered. The somewhat high percentage of sulfate recovered when 2 cc. portions of the filtrates were analyzed suggested the possibility that sulfate, like the total base of serum (17) or the sodium of erythrocytes (18), might be unequally distributed between coagulum and filtrate when trichloroacetic acid is employed to precipitate protein, so that the filtrate contained the higher concentration of sulfate. To test this possibility, serum containing added sulfate was deproteinized in dilutions of 1:2 and 1:4, that is with an equal volume of 20 per cent and with 3 volumes of 12 per cent trichloroacetic acid, respectively, after which 1 cc. portions (plus 1 cc. of 8.7 per cent trichloroacetic acid) of the filtrate representing the 1:2 dilution and 2 cc. portions of the filtrate representing the 1:4 dilution were precipitated with alcoholic benzidine. The results of these analyses (four experiments) agreed closely, within less than 1 per cent, indicating that, within the limitations of the method of analysis, sulfate is not present in the protein-free filtrate in greater concentration than in the coagulum.

Letonoff and Reinhold (19) have stated, in connection with the use of their new method for the determination of sulfate, that sulfate appears to become bound in some manner, and that the recovery of added sulfate was only 77 to 40 per cent complete if the analysis was delayed 5 to 10 minutes or longer after addition of the sulfate to the serum. The data in Table III show that such large losses have not been encountered in our work. This question was investigated further by beginning the analyses earlier in several experiments; that is, within 5 minutes after the addition of sulfate to serum. The results of these analyses when compared with the results of analyses of the same mixtures initiated 30

TABLE III  
*Recovery of Sulfate Added to Serum*

The values for sulfate are expressed as mg. of S in 100 cc. of serum.

Experi- ment No.	Serum	Inorganic sulfate present	Sulfate added	Filtrate precipi- tated	Total sulfate found	Sulfate recov- ered	Sulfate recov- ered
				cc.			per cent
1	Human	0.870	1.384	1	2.160	1.290	93.2
				2	2.230	1.360	98.3
2	"	1.037	1.384	1	2.367	1.330	96.1
				2	2.537	1.500	108.4
3	"	1.080	1.384	1	2.370	1.290	93.2
				2	2.467	1.387	100.2
4	"	1.090	1.384	1	2.400	1.310	94.6
				2	2.517	1.427	103.1
5	"	1.100	1.384	1	2.457	1.357	98.1
				2	2.533	1.433	103.5
6	"	1.120	1.384	1	2.463	1.343	97.0
				2	2.607	1.487	107.5
7	"	1.127	1.384	1	2.480	1.353	97.7
				2	2.563	1.436	103.8
8	"	1.213	1.384	1	2.567	1.354	97.8
				2	2.633	1.420	102.6
9	"	1.313	1.384	1	2.750	1.437	103.9
				2	2.770	1.457	105.3
Average				1			96.8
				2			103.6
10	Human	1.05	2.57*	2	3.730	2.680	104.3
			2.57†	2	3.700	2.650	103.0
11	Dog	3.27	1.75*	2	5.110	1.840	105.0
		3.17†	1.75*	2	4.990†	1.820	104.0
12	Horse§	2.970	3.17*	2	6.140	3.170	100.0
			3.17†	2	6.170	3.200	101.0
13	" §	3.73	3.17†	2	6.900	3.170	100.0

\* Serum plus added sulfate stood 5 minutes before precipitation with trichloroacetic acid.

† Serum plus added sulfate stood 30 minutes before precipitation with trichloroacetic acid.

‡ Microgravimetric determinations.

§ 1 volume of serum was deproteinized with 4 volumes of 11.5 per cent trichloroacetic acid.

minutes after addition of the sulfate (Experiments 10 to 13, Table III) show that the time the mixtures were allowed to stand within the limits stated had no significant influence on the recovery of added sulfate. Human serum, dog serum, and horse serum were used for these comparisons.

*Total and Ethereal Sulfates*—The quantity of sulfate present in filtrates of human serum that have been subjected to acid hydrolysis, that is the total sulfate, has been found to be but slightly

TABLE IV  
*Inorganic, Total, and Ethereal Sulfate in Human Serum*  
The values for sulfate are expressed as mg. of S in 100 cc. of serum.

Serum No.	Inorganic sulfate	Total sulfate	Ethereal sulfate	Ethereal sulfate, per cent of total sulfate
1	1.05	1.05	0.00	0.0
2	1.02	1.04	0.02	1.9
3	0.94	0.97	0.03	3.1
4	1.06	1.10	0.04	3.6
5	1.21	1.26	0.05	4.0
6	1.13	1.19	0.06	5.0
7	1.35	1.42	0.07	5.0
8	1.14	1.20	0.06	5.0
9	1.24	1.31	0.07	5.3
10	1.49	1.58	0.09	5.7
11	1.28	1.36	0.08	5.9
12	1.05	1.13	0.08	7.1
13	1.09	1.19	0.10	8.4
14	1.28	1.40	0.12	8.6
15	1.28	1.46	0.18	12.3
16	1.14	1.33	0.19	14.3
Average . . .			0.076	6.0

greater than the inorganic fraction. Consequently, the calculated values for ethereal sulfate are small, and range from 0.0 to 0.19 mg. of S per 100 cc. of serum, the average being 0.076 mg. (Table IV). In terms of the fraction of the total sulfate, these data correspond to 0.0 to 14.3 per cent; average, 6 per cent. Obviously, the determination of such a small fraction cannot be attended with great accuracy. DeMeio (20) recently has reported much higher values for total sulfate in normal human serum than we have



found; namely, 1.46 and 1.75 mg. of S per 100 cc. in two instances and 3.17 to 4.0 mg. per 100 cc. in eight other instances. We are unable to accept such high values, and suggest that the conditions selected by DeMeio for precipitation of sulfate with benzidine and acetone after acid hydrolysis of his filtrates lead to unsuspected errors. Reed and Denis (21) in their earlier work with the nephelometric barium sulfate method likewise found much higher values for total sulfate, and hence the ethereal sulfate fraction, than we have encountered. In this instance also we suspect that the method of determination was inadequate.

*Range of Normal Values*—The patients studied were selected after consideration of the clinical histories and the results of physical examination had indicated the absence of renal insufficiency or other abnormalities likely to influence the concentration of inorganic sulfate in the serum. In each instance blood was drawn in the morning, before breakfast. The mean value for the serum sulfate in this group (twenty-nine men ranging in age from 17 to 61 years) was 1.11 mg. of S per 100 cc. and the standard deviation was  $\pm 0.13$  mg. The values ranged from 0.87 to 1.47 mg. of S per 100 cc. and twenty (69 per cent) of the twenty-nine determinations differed from the mean by an amount less than or equal to the value of the standard deviation. The distribution of the values was therefore normal. These figures are comparable in magnitude to those of Loeb and Benedict (22), who used the gravimetric barium sulfate method of analysis. They reported that the amount of sulfate in normal serum (thirteen determinations) ranged from 0.7 to 1.6 mg. of S per 100 cc. and that the average amount was 1.13 mg. per 100 cc. Øllgaard (23), who likewise employed a method that involved the use of barium, reported that the range of normal values, according to his technique, was 0.70 to 1.50 mg. of S per 100 cc. in a series of twenty specimens of normal serum.

We believe the data given by these investigators, together with the data presented in this paper, represent the best available values for the concentration of inorganic sulfate in normal human serum. Hoffman and Cardon (24) have reported that the values for the concentration of inorganic sulfate in normal human serum, as determined by their method, range from 0.34 to 1.09 mg. of S per 100 cc. of serum. We believe that these values are almost cer-

tainly too low and we were unable to obtain satisfactory recovery of sulfate when inorganic solutions or dialyzed serum containing added sulfate were subjected to their procedure for deproteinization. The values reported by Letonoff and Reinhold (19), 0.95 to 1.16 mg. of S per 100 cc. of serum, with an average of 1.04 mg. (twenty-three normal persons), agree rather well with the results of our studies; nevertheless, the difficulty which these authors encountered in obtaining a satisfactory recovery of sulfate which had been added to serum suggests that the data obtained by their method should be accepted with some caution.

#### SUMMARY

A method for the determination of sulfate in serum is described, in which the sulfate is precipitated from a trichloroacetic acid filtrate by the addition of a solution of benzidine in 95 per cent alcohol. Benzidine sulfate precipitated in this manner possesses a characteristic white silky appearance and is associated with considerably fewer impurities than when a solution of benzidine in acetone is used as the precipitating agent. The recovery of known amounts of sulfate from solutions containing the inorganic constituents that would be present in a trichloroacetic acid filtrate of serum is likewise improved by the use of alcohol instead of acetone.

Values found for the amount of sulfate in serum filtrates by the method described were in reasonable agreement with the results of determinations made by the microgravimetric method. The degree of recovery of sulfate added to serum was satisfactory, and there was no evidence that a part of the added sulfate was lost by becoming bound in some manner.

The differences between the results of determinations of inorganic and total sulfate in serum filtrates indicate that the ether-soluble sulfate fraction is small, of the order of 5 to 10 per cent of the total. This is considerably less than has been reported by other investigators.

In a group of twenty-nine normal men, the values for the inorganic sulfur of the serum ranged from 0.87 to 1.47 mg. per 100 cc. of serum; the mean value was 1.11 mg. per 100 cc., and the standard deviation was  $\pm 0.13$  mg.

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## MODE OF ACTION OF PANCREATIC LIPASE\*

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In the past the specificity of lipase action for fats has been to some extent taken for granted. Pancreas extracts were known to hydrolyze all the ordinary glycerides (except possibly the stearins) and this behavior defined the enzyme lipase. There have been good reasons for supposing that stearins constituted no exception in principle, but were merely attacked very slowly. Actually, tristearin was split so poorly by otherwise powerful preparations of pancreas lipase that its digestion, when observed at all, was entirely out of line with what might have been expected for a commonly occurring edible fat.

We have recently shown, however, that the hydrolysis of mono-, di-, or tristearin can be carried out at a rate quite comparable to that found with the other fats, provided special conditions are maintained involving a high temperature and a good emulsion (1). The temperature of digestion is best kept at 40° or over. The emulsion is made by dissolving the stearin in a hot mixture of bile and glycerol, cooling down the solution until it is pasty, and then diluting with the required buffer.<sup>1</sup> This technique, as proved by the rapid digestion of tristearin, ought to give a more complete picture of the range of lipase activity on other substrates than has hitherto been obtainable.

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<sup>1</sup> An explanation of the effect of increased temperature is perhaps found in the work of Clarkson and Malkin (2), who have shown that the internal arrangement of higher glycerides is affected by heating. It is not improbable that such changes in molecular configuration can produce a form of the substrate vulnerable to lipase. The lower glycerides may already be a mixture of several forms in equilibrium.

We have, therefore, experimented with the action of lipase on a number of esters which could not perhaps have been fairly tested by other methods, and whose splitting, so far as we are aware, has not been previously observed. These attempts have been justified by the finding that many esters of higher fatty acids other than the glycerides are saponified by crude lipase at a rate of the same order of magnitude as that shown by the saturated fats.

Most of the substrates tested throughout the work have been synthesized or at least purified in this laboratory.<sup>2</sup> In some instances well known commercially available chemicals were used after testing. No attempt was made at this time to purify the crude glycerol extract of pig pancreas employed as an enzyme preparation. Control experiments, however, eliminated the possibility that the observed hydrolyses were due to the digestion of protein or other constituents of the enzyme system, or to the spontaneous decomposition of the substrates.

#### *Effect of Fatty Acid Component of Ester on Its Rate of Hydrolysis*

Pancreas extracts hydrolyze all the triglycerides of *n* saturated fatty acids. Respectively, however, they are split at very different rates, as shown in Fig. 1. The fats that are split most rapidly are the glycerides of the fatty acids occurring midway between acetic acid and stearic acid. The possibility exists, however, that such results merely portray the degree of emulsification attained. Nevertheless, a similar experiment with various esters of a monohydric alcohol shows the same relationship. The lipase is best adapted to the splitting of esters of fatty acids of intermediate chain length, whether the esters in question are triglycerides or esters of ethyl alcohol.

In no case do the ethyl esters hydrolyze as rapidly as the tri-

<sup>2</sup> We have not found records of the following compounds in the literature: tertiary butyl stearate, m.p. 33°; neopentyl stearate, m.p. 35°; tertiary amyl stearate, m.p. 27.5°; and secondary propyl stearate, m.p. 25°. They were all made without difficulty by the well known quinoline method with stearyl chloride and the appropriate alcohol. They all crystallized readily. We are indebted to Professor F. C. Whitmore for the neopentyl alcohol. Melting points differing materially from those recorded in Beilstein were observed for isobutyl stearate, found to melt at 28.5° instead of at 25° as recorded, and for benzyl stearate, found to melt at 45° instead of at 28° as recorded.

glycerides, but the difference in speed is not very startling. Even ethyl palmitate and ethyl stearate are split rapidly. It is also evident that a separate esterase, at least as found in the liver, plays no significant part in the hydrolyses reported here, for the reason that horse liver extract rich in esterase (splitting ethyl butyrate) was without effect on the stearates used in our experiments.

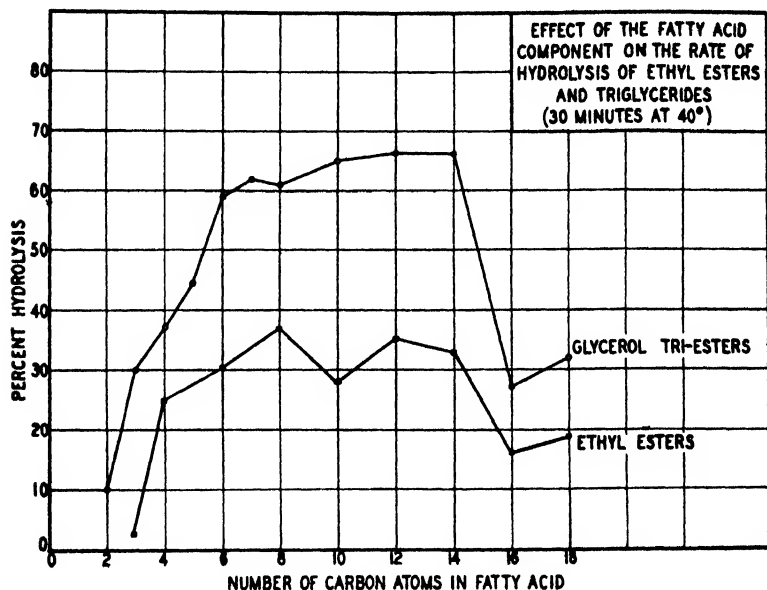


FIG. 1

The specificity of the liver system as contrasted to that of the pancreas is shown in Table I. In general, both liver and pancreas extracts split the lower esters (such as the butyrates) of a variety of alcohols, but only the pancreas enzyme splits the stearic esters. A differentiation noted by Loevenhart (3) between the substrates of pancreas enzyme and liver enzyme is clearly shown in Table I by the hydrolysis of the triglycerides. Liver extract hydrolyzed tributyrin very rapidly, trivalerin slowly, and tricaproin practically not at all. The upper limit of the liver esterase is here the fat of the straight chain  $C_5$  acid.

TABLE I  
*Action of Liver and Pancreas Extracts on Several Esters*

Substrate	Concentration	Per cent splitting in 2 hrs.*	
		Liver extract	Pancreas extract
	<i>mM</i>		
Ethyl butyrate.....	0.2	95	55
“ propionate .....	0.2	72	15
“ myristate.....	0.2	6 (18 hrs.)	115†
Methyl stearate.....	0.2	1.3 (18 “ )	64
Ethyl “ .....	0.2	3.2 (18 “ )	45
Propyl “ .....	0.2	-1.0 (18 “ )	46
Allyl “ .....	0.2	-1.9 (18 “ )	85
Butyl “ .....	0.2	3.2 (18 “ )	75
Isobutyl stearate.....	0.2	0 (18 “ )	118†
Isoamyl “ .....	3.2	0 (18 “ )	91
Neopentyl “ .....	0.2	4.7 (18 “ )	23 (18 hrs.)
Benzyl stearate.....	0.2	4.6 (18 “ )	101
Cetyl “ .....	0.2	10.7 (18 “ )	23
Cholesterol stearate....	0.2	0 (18 “ )	0.0 (18 hrs.)
Secondary propyl stearate.	0.2	5.5 (18 “ )	9 (18 “ )
“ butyl “ .....	0.2	0.4 (18 “ )	13 (18 “ )
Tertiary “ “ .....	0.2	4.7 (18 “ )	±3 (18 “ )
“ amyl “ .....	0.2	0 (18 “ )	4 (18 “ )
Ethylene glycol dibutyrate	0.2	66	56
Trimethylene glycol dibutyrate.....	0.2	71	57
Ethylene glycol dicaprate.	0.1		99 (30 min.)
“ “ distearate..	0.1	-0.5 (18 hrs.)	2.1 (14 hrs.)
Trimethylene glycol distearate.....	0.1	1.3 (18 “ )	27
Propylene glycol monostearate.....	0.2	-0.5 (18 “ )	37

\* The amount of substrate as stated in the table plus 0.33 cc. of glycerol extract per titration. Temperature 40°.

† Because with some esters a part of the undigested substrate is apt to cling to the glass container during the digestion, aliquot portions of the liquid removed for titration toward the end of an experiment may contain more than their proportionate share of free acid. The end-results may therefore show more digestion than they should. This error is not important for a qualitative interpretation of the results, but it must be considered in any experiment designed to differentiate between complete and incomplete splitting. It may be avoided by titrating the entire contents of a container at each time interval.

TABLE I—*Concluded*

Substrate	Concentration	Per cent splitting in 2 hrs.*	
		Liver extract	Pancreas extract
	<i>mM</i>		
Tripropionin.....	0.2	33	44
Tributyryn.....	0.2	" 33	59
Trivalerin.....	0.1	62	70
Tricaproin.....	0.2	1.5	73
Trimyristin.....	0.1	0.8 (18 hrs.)	91
Tripalmitin.....	0.1	-1.0	83
Tristearin.....	0.1	1.3	80
Triisovalerin.....	0.1	30	30

*Effect of Alcohol Component of Ester on Its Rate of Hydrolysis*

This has been examined with a fairly long list of stearic acid esters, and the observed velocities of hydrolysis under comparable conditions are exhibited in Fig. 2. The results show a surprising indifference on the part of the enzyme to the constitution of the alcohol, as long as it is of the primary variety. The length of the carbon chain, the occurrence of free hydroxyl groups, the presence of a double bond or of secondary or tertiary carbon atoms may all affect the rate of splitting, but do not change the qualitative or even the general quantitative aspect of the result.<sup>3</sup> It is evident then that the configuration of the alcohol is without effect on the enzyme, except in so far as the configuration of the hydroxyl-bearing carbon atom is concerned.

On the other hand the splitting of secondary and tertiary esters occurs with great difficulty. The results obtained with secondary propyl stearate, secondary butyl stearate, tertiary butyl stearate, and tertiary amyl stearate are in marked distinction to those with the corresponding esters of *n*-propyl, *n*-butyl, isobutyl, *n*-amyl, and isoamyl alcohols. The splittings observed with these refractory substances were barely greater than the maximum experimental error over a period of 18 hours at 40°. The slight hydrolysis may possibly be due to a trace of primary alcohol in the

\* It must be remembered that small differences in speed are probably due to differences in the fineness of the emulsion which, in spite of the best efforts, must vary from one substance to another.



material used for the ester synthesis. It is therefore difficult to say whether these substances are split very slowly or not split at all. It is safe to assume, however, that the splitting of a secondary ester linkage takes place at an almost negligible velocity, in comparison with the splitting of a primary ester linkage.

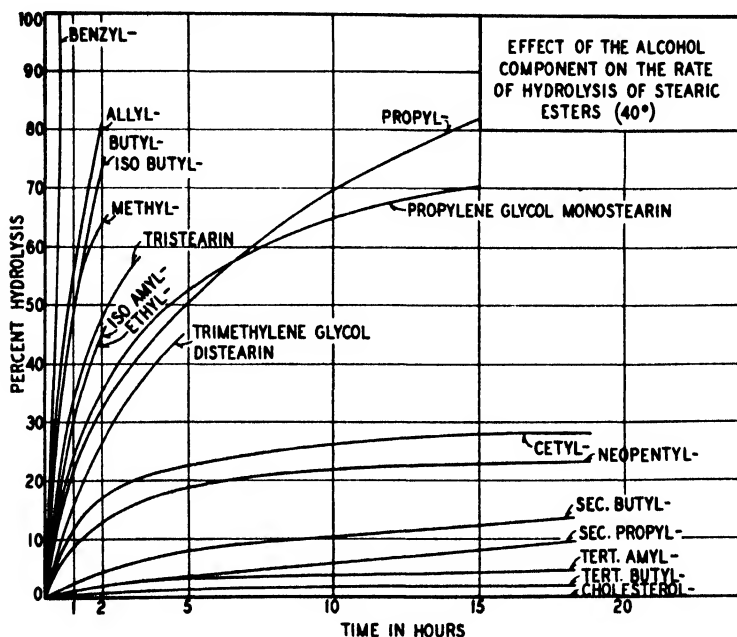


Fig. 2

## DISCUSSION

The liver esterase is known to be distinguished from the pancreas enzyme by its specificity for esters of the lower fatty acids, whether esters of glycerol or of simple monohydric alcohols. The pancreas enzyme, however, has been described as digesting the higher fats only. It is now apparent by the use of more inclusive methods that the higher esters of primary monohydric alcohols are also vulnerable. Our data do not indicate whether pancreas lipase also splits the lower esters attacked by the liver enzyme, or whether this was due to an accompanying esterase. The net

result, however, was that crude pancreas extract rapidly hydrolyzed all the primary esters tried except ethylene glycol distearate and dipalmitate.

The anomalous behavior of these two glycol esters might seem very significant, were it not for the fact that until the present technique was adopted, tristearin was found as resistant to lipase action as these esters now appear to be. Further attempts to split them therefore seem advisable before drawing any conclusions. It may in any event be pointed out that while all primary esters have not been hydrolyzed by lipase, primary esters only have been split.

The configuration of the ester-bearing carbon atom of the alcohol must therefore be of great importance to the action of lipase. Either the enzyme requires 2 haptophore hydrogen atoms on this carbon atom or else the replacement of one of these hydrogens by a larger group prevents the approach of the enzyme. The latter view seems more probable because there is no independent evidence to show that the hydrogen atoms in question are specially active. In either case, however, the alcoholic carbon atom of the ester linkage or its neighboring alcoholic oxygen atom is clearly indicated as an anchoring point of the lipase. -

The conclusion that only primary ester linkages can be split with reasonable velocity by lipase is of importance in considering the mechanism of enzymic fat hydrolysis. The fats contain one secondary ester linkage, yet are split rapidly and completely. Complete splitting can therefore occur only if the secondary ester group in a triglyceride possesses properties essentially different from the secondary groups in the substrates reported here, or if the migration of the  $\beta$  ester group to an  $\alpha$  position takes place before it is hydrolyzed. Further experiments aided by the cooperation of Professor C. G. King of the University of Pittsburgh are in progress on this point.

#### SUMMARY

An investigation of the effect of pancreas lipase on a series of monoesters shows that ethyl esters of the higher fatty acids are split almost as easily as are the fats themselves. The rate of splitting of a series of ethyl esters exhibits the same characteristic variation with the length of the fatty acid carbon chain as was observed with a series of corresponding triglycerides.

A change in the alcohol component of the ester materially affects the rate of hydrolysis only when the configuration of the carbon atom bearing the hydroxyl group is concerned. Evidence is presented pointing to the conclusion that only primary ester groups are split by lipase. One point of attachment for the enzyme on the substrate would therefore appear to be the C—O— on the alcohol side of the ester linkage. It also follows that either the  $\beta$  ester group in a fat has entirely different properties from those of other secondary esters, or else enzymic hydrolysis must be accompanied by a migration of the  $\beta$  group to an  $\alpha$  position.

The authors wish to acknowledge their indebtedness to Mr. I. W. Tucker, of this laboratory, for his assistance throughout the work.

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## THE PREPARATION OF CITRULLINE BY HYDROLYSIS OF ARGININE

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(Received for publication, February 7, 1938)

The need for 50 gm. of citrulline for the thermal data being compiled in this laboratory (1, 2) led to the search for a suitable method of preparation of this compound. Of the methods available, the most practical were Kurtz's ingenious three reaction degradation of arginine (3), and Wada's tryptic digestion of casein (4). Each of these authors, however, reported the preparation of less than 2 gm. of citrulline, and neither method is of the type to lend itself readily to the processing of larger batches. The necessity for troublesome barium hydroxide and sealed tube manipulations in one case, and a 3 week incubation in the other, increased the desirability of a rapid and simple process.

Although the biological conversion of arginine to citrulline is well known (5, 6), these reactions are inconvenient as preparative procedures. The alkaline hydrolysis of arginine has been shown to give ornithine (7, 8), urea (9), and carbon dioxide and ammonia (10). The suggestion that citrulline might similarly be obtained has been offered, and at least one such attempt has been recorded (3, 5, 11). The hydrolysis of arginine may be represented as follows:



The present author has, under proper conditions, consistently isolated copper citrullinate from the alkaline hydrolysis of arginine solutions. This discrepancy with former work may be due to the fortunate finding of an effective means of isolation. A more likely explanation, however, is that the previous authors, in using a large

molar excess of alkali, caused rapid decomposition of any citrulline formed. To test this, in the present investigation, 10.0 mm of arginine were refluxed with 180 mm of sodium hydroxide solution until 11.5 mm of ammonia were evolved, yet no copper citrullinate could be separated. The possibility that this failure was due to the altered character of the reaction mixture is not to be excluded with finality. At any rate, successful hydrolysis of arginine to citrulline is readily accomplished by a 1:1 M ratio of sodium hydroxide to arginine.

The hydrolysis of arginine, as described in the experimental part, has been run in a single batch to give 65 gm. of citrulline; larger runs can probably be managed equally well. The operations are few and simple, and the yield, based on protein employed, is as good as that of any of the previous methods. A further important advantage is that arginine hydrochloride can be hydrolyzed and several gm. of free citrulline obtained in the same day.

The hydrolysis of arginine to citrulline is the reverse of their relationship in the urea scheme of Krebs and Henseleit (12). A closer analogy is to be found in the work of Bell (13), who obtained urea in good yield from the hydrolysis of guanidine with an equimolar quantity of base, whereas an excess of alkali was known to give carbon dioxide and ammonia (14). Bell found that free guanidine solutions were sufficiently alkaline of themselves to form urea on boiling. Attempts to obtain an analogous result with free arginine, in this work, failed to yield appreciable amounts of ammonia, as did boiling with an equimolar quantity of 0.1 N alkali. Normal alkali was, however, effective, and the more concentrated sodium hydroxide finally employed proved even more satisfactory.

#### EXPERIMENTAL

198 gm. of arginine hydrochloride (0.938 mole), prepared from U.S.P. gelatin by the method of Brand and Sandberg (15), were placed in a 1 liter round bottom flask with 330 ml. of 5.68 N sodium hydroxide (1.876 moles, 0.938 mole for neutralization of hydrochloride). The flask was fitted with a condenser and attached tube leading to slightly above the surface of a quantity of standard hydrochloric acid. The arginine solution, with glass

## S. W. Fox

beads, was brought carefully to boiling, and refluxed  $3\frac{1}{2}$  hours. At the end of this time, 0.64 mole of ammonia had been evolved. The cooled solution was acidified with glacial acetic acid and evaporated under reduced pressure on the hot water bath to approximately 250 ml., 300 ml. of water were added, and the evaporation was repeated. The residue was treated with 1500 ml. of absolute ethanol, and the liquid poured off after separation was complete. The crude citrulline was washed with further small quantities of alcohol and then dissolved in 800 ml. of water.

The solution was boiled 30 minutes with 79 gm. of copper oxide, and the copper citrullinate and unchanged copper oxide were filtered off.<sup>1</sup> The filtrate was evaporated down over a steam bath, and successive crops were removed by filtration. Four such crops were combined with the residue from the original filtration and the entire batch was washed thoroughly with water until the washings were colorless. A sample recrystallized from water decomposed at  $257-258^{\circ}$  (corrected).<sup>2</sup> The copper salts were suspended in 1 liter of water, which was then saturated with hydrogen sulfide. The copper sulfide was coagulated by boiling, filtered hot, and the filtrate was cleared with norit. The solution was evaporated under reduced pressure on the hot water bath until crystals began to appear and was then treated with several volumes of absolute ethanol. The separated crystals were dried *in vacuo* over phosphorus pentoxide to give 65 gm. of white citrulline.

A sample was recrystallized from water and alcohol.

$C_6H_{13}N_3O_8$ .	Calculated.	Amino N	7.99
	Found.	" "	7.94, 7.89 (microformol)

When examined in aqueous solution, and in dilute hydrochloric acid, the citrulline showed no optical activity.

### SUMMARY

The theoretical hydrolysis of arginine to citrulline *in vitro* has been accomplished and discussed. The details for rapidly and

<sup>1</sup> In the smaller, pilot runs, no copper citrullinate crystallized at this stage, and it was possible to filter off copper oxide alone.

<sup>2</sup> The decomposition point of the copper salt is highly reproducible, whereas for the free citrulline values from  $202-226^{\circ}$  are recorded in the literature, and ranges in the interval  $200-218^{\circ}$  were found in this work.

conveniently preparing a 65 gm. quantity of citrulline with the aid of this single reaction have been presented.

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## THE ESSENTIAL NATURE OF ARGININE IN THE DIET OF THE CHICK

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Arnold, Kline, Elvehjem, and Hart (1) have reported that arginine is necessary for the rapid growth of the chick. Their experiments demonstrated that the growth of chicks on a ration containing 12 per cent casein could be increased by supplements rich in arginine or by pure arginine.

Rose and his coworkers (2, 3) have shown that histidine but not arginine is necessary for growth of the rat. Later Rose (4) stated that exclusion of arginine from the diet causes a decrease in the growth rate to about three-fourths of the normal value.

The purpose of the present work was to test the indispensability of tryptophane, histidine, and arginine, with diets which were very low in these amino acids. In addition, the adequacy of whole casein as a source of arginine was investigated.

### EXPERIMENTAL

*Composition of Diets*—All of the experimental diets contained the following: 40 per cent polished rice, 19 per cent starch (9 per cent when 30 per cent casein was present), 10 per cent soy bean oil, 1 per cent cod liver oil, 3 per cent salt mixture (5), 0.05 per cent manganous sulfate, 3 per cent liver extract, 2 per cent crude milk salts, 2 per cent Galen B Type II, and 0.2 per cent cystine. The rice proteins contributed a small amount of arginine which we have estimated at not more than 0.2 per cent of the diet. The remainder of the diet, consisting of casein and amino acid supplements, is listed individually for each experiment in Tables I and II.

The liver extract was the 70 per cent alcohol-soluble portion of a hot water extract of freshly killed beef liver, 1 gm. of this concentrate being equivalent to 14 gm. of fresh liver.



The crude milk salts concentrate was the 95 per cent alcohol extract of a concentrated wash water from crude milk salts, the

TABLE I

*Composition of Diets Used in Experiments I and II*

Diets 145 to 150 inclusive were fed to 10 day-old chicks with an average weight of 72 gm., Diets 152 and 153 to 14 day-old chicks with an average weight of 86 gm., Diets 154 to 162 to 14 day-old chicks with an average weight of 103 gm.

Experiment No.	Diet No.	Amount and type of casein	Tryptophane added to diet	Histidine added to diet	Arginine monohydrochloride added to diet	Time on diet	Average gain per chick per day	Average weight of feed consumed per chick per day
			per cent	per cent	per cent	days	gm.	gm.
I	145	20% acid-hydrolyzed				13	-0.7	3.2
	146	Casein III	0.2			13	+2.3	8.6
	147	20% acid-hydrolyzed	0.2			13	-0.9	3.2
	148	Casein I, histidine- and	0.2	0.5		13	-1.1	2.9
	149	arginine-free	0.2		1.0	13	-0.1	3.7
	150		0.2	0.5	1.0	13	+4.5	11.1
	152	20% common casein				14	+5.6	12.0
	153				0.5	14	+7.7	14.8
	154	20% acid-hydrolyzed	0.2			10	-1.9	3.6
	155	Casein II, arginine-free	0.2	0.5		10	-1.6	4.0
II	157		0.2	0.5	1.0	10	+2.7	10.0
	158		0.2	0.5	1.8	8	+2.5	9.8
	159	20% common casein				10	+4.8	10.8
	160				0.5	10	+7.3	14.5
	161	30% " "				10	+6.3	11.2
	162				0.5	10	+6.5	11.7

wash water at the time of extraction having a solids content of 60 per cent.

All of the amino acid supplements, with the exception of the

histidine used in Experiments I and II and the tryptophane in Experiment I, were commercial products. The tryptophane used in Experiment I was prepared from a tryptic digest of casein, the crude product obtained from the butyl alcohol extraction being purified by recrystallization from 70 per cent alcohol.

TABLE II

*Composition of Diets Fed 15 Day-Old Chicks with an Average Weight of 105 Gm. (Experiment III)*

Diet No.	Amount and type of casein	Tryptophane added to diet	Histidine monohydrochloride added to diet	Arginine monohydrochloride added to diet	Ornithine dihydrochloride added to diet	Urea added to diet	Time on diet	Average gain per chick per day	Average weight of feed consumed per chick per day
		per cent	per cent	per cent	per cent	per cent	days	gm.	gm.
163	20% acid-hydrolyzed Casein II, arginine-free	0.2	0.5				11	-1.5	5.2
164		0.2	0.5	1.0			11	+4.1	11.6
165		0.2	0.5		1.0		11	-1.5	4.6
166		0.2	0.5			1.0	11	-1.5	4.1
167		0.2	0.5		1.0	1.0	11	-1.8	5.4
172	20% acid-hydrolyzed Casein III	0.2					9	+3.9	12.0
168	20% common casein						9	+4.5	13.2
169				0.5			9	+8.1	14.3
170	30% " "						9	+7.0	12.5
171				0.5			9	+7.2	13.6

*Preparation of Hydrolyzed Casein*—2 kilos of casein were hydrolyzed by refluxing on a sand bath for 17 hours with 6 liters of concentrated hydrochloric acid. The hydrochloric acid was removed by the addition of 750 cc. of concentrated sulfuric acid and concentration of the resulting solution *in vacuo*. One-half of this final concentrate, equivalent to 1 kilo of casein, was used for

the following preparation of a histidine- and arginine-free hydrolyzed casein.

The histidine was precipitated with silver oxide at pH 7.2 and the precipitate filtered off and washed twice with water. The filtrate and washings were combined, acidified with sulfuric acid to pH 3.8, and concentrated in preparation for the removal of arginine. The histidine precipitate was dissolved with sulfuric acid (pH 5.0), filtered, and reprecipitated by adding barium hydroxide to pH 7.3.

The crude histidine fraction was purified by decomposition of the silver salt with hydrogen sulfide, precipitation of the mercury salt, and subsequent decomposition of it with hydrogen sulfide, the procedure of Vickery and Leavenworth (6) being followed in general. The histidine obtained from the decomposition of the mercury salt was recrystallized from alcohol-water solution and was used as the histidine supplement in Experiments I and II.

The concentrated histidine-free hydrolysate was freed of arginine by the use of flavianic acid, as described by Bunney and Rose (3). The dried hydrolysate, free of histidine and arginine, was designated acid-hydrolyzed Casein I.

The arginine-free acid-hydrolyzed casein used in Experiments II and III and called acid-hydrolyzed Casein II was prepared according to the procedure of Bunney and Rose. This product was not necessarily deficient in histidine; however, histidine supplements were used in Experiments II and III to insure against any possibility of a histidine deficiency.

The acid-hydrolyzed Casein III used in Experiments I and III was hydrolyzed with sulfuric acid, the sulfuric acid was removed with barium hydroxide, and the hydrolysate dried. All of the acid-hydrolyzed caseins were deficient in tryptophane owing to its destruction in acid solution.

*Care of Chicks*—The newly hatched, single comb, white Leghorn chicks were placed in metal, wire-floored, electrically heated, battery brooders and kept on a standard chick mash for approximately 2 weeks. At this time they were wing-banded, weighed, and segregated into groups of seven each, each group having the same weight distribution and average weight. The groups were then placed on experimental diets. The chicks were weighed individually and the total feed consumption of each group was noted

at 2 to 4 day intervals for the duration of the experiment. Feces were collected for urea determinations from chicks on Diets 163 to 167 inclusive.

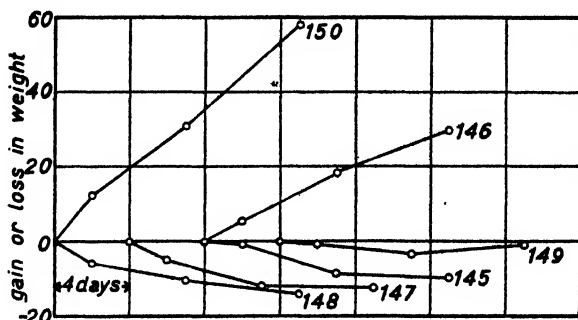


FIG. 1. Growth curves showing average gain or loss of weight in gm. of chicks on diets in Experiment I.

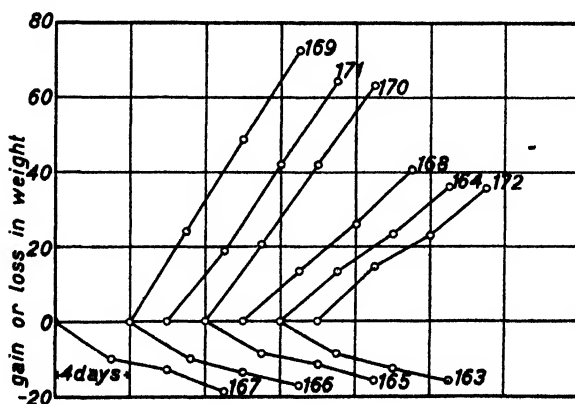


FIG. 2. Growth curves showing average gain or loss of weight in gm. of chicks on diets in Experiment III.

### Results

The weight changes after 2 or 3 days of feeding were in all cases completely indicative of the final results at the end of the feeding period. The weight responses and feed consumption data for the complete feeding period are given in Tables I and II. Figs. 1 and 2 show how consistent the weight changes were throughout the period.

Experiment I indicates the indispensability of tryptophane, histidine, and arginine for growth of the chick. The essential nature of arginine in the diet was again demonstrated in Experiments II and III. The failure of the group on Diet 158, receiving 1.8 per cent arginine monohydrochloride, to gain more than the group on Diet 157 would seem to indicate that 1 per cent arginine monohydrochloride furnished sufficient arginine for the optimum growth of the chick on this diet.

In Experiment III, the possibility that the chick is able to synthesize arginine from ornithine, urea, or a combination of these two compounds was investigated. The results were completely negative.

Weight responses to diets containing 20 and 30 per cent casein, both with and without the 0.5 per cent arginine monohydrochloride supplement, furnished several interesting facts. 20 per cent casein was inadequate for the chick with respect to available arginine and was beneficially supplemented by arginine monohydrochloride. 30 per cent casein contained an adequate amount of arginine, since supplementing this level of casein with arginine had no further effect. The two groups on the 30 per cent casein level, Diets 161 and 162 in Experiment II and Diets 170 and 171 in Experiment III, showed somewhat poorer growth than the arginine-supplemented groups given 20 per cent casein.

The amount of urea excreted, on the basis of mg. per chick per day per gm. of feed consumed, was 1.9 for Diet 163, 1.7 for Diet 164, 1.8 for Diet 165, 2.7 for Diet 166, and 2.2 for Diet 167. The first three diets evidently fall into one group, while the last two diets, supplemented with urea, cause somewhat higher urea excretion. The amount of urea recovered in the droppings was only a small fraction of the amount consumed.

#### DISCUSSION

From the results of these experiments, it is evident that the chick is not able to synthesize sufficient arginine for maintenance of body weight, even when supplied with ornithine. The urea excretion in terms of mg. of urea per chick per day per gm. of feed consumed was essentially the same when the diet was deficient in arginine, or contained supplementary arginine, or contained ornithine. The chick obviously lacks the mechanism of arginine formation and decomposition which exists in mammals.

The inability of the chick to synthesize arginine from ornithine is in harmony with the observations of Needham, Brachet, and Brown (7), who have found that 5 day-old chick embryos, *in vitro*, were able to form urea from added arginine, but were not able to produce urea when ornithine and ammonium chloride were added.

The report of Crowdle and Sherwin (8) that the bird, on an arginine-low diet (polished rice, potatoes, and yeast), can synthesize ornithine for the detoxication of benzoic acid is interesting in view of the inability of the bird to synthesize arginine. Accompanying the detoxication product, ornithuric acid, there was a 5- to 20-fold increase in the urea excretion. Since arginase is present in small amounts in the kidney of the bird, the extra urea production may indicate that the ornithine was actually derived from arginine of the tissues or from the diet, which undoubtedly contained a small amount of arginine. This explanation has also been advanced by Hunter and Dauphinee (9).

The failure of the diets containing 20 per cent casein to satisfy the arginine requirements of the chick raises the question of the availability of arginine in casein. In experiments with arginine-free acid-hydrolyzed casein, 1 per cent arginine monohydrochloride, equivalent to 0.83 per cent arginine, gave sufficient arginine for the maximum growth which can be expected from acid-hydrolyzed casein diets, since increasing the arginine to 1.5 per cent gave no better growth. The diets with 20 per cent casein furnished 0.77 per cent arginine from casein, if the percentage of arginine in casein is taken as 3.85, and not more than 0.2 per cent arginine from the rice protein, or a total of 0.97 per cent arginine. These sources of arginine were inadequate and supplementing with arginine gave strikingly better growth. Evidently part of the arginine in casein exists in such a form as to be unavailable to the chick. This corresponds to the finding of Dauphinee and Hunter (10) that complete tryptic digestion of casein results in the liberation of only 80 per cent of the total arginine.

It is interesting that the diets containing 30 per cent casein, both supplemented and unsupplemented, produced growth inferior to that on the arginine-supplemented diet with 20 per cent casein. From this it would seem that, above 20 per cent casein in the diet, the additional casein had no beneficial effect other than increasing the amount of available arginine. The results, in fact,

indicate that the extra casein in the diet exerted a slight retarding effect on growth, but this retardation cannot be linked with an arginine deficiency.

It may also be pointed out that the most favorably supplemented acid-hydrolyzed casein diets permitted a rate of growth which was inferior to that on diets containing 20 per cent un-supplemented common casein and only approximately one-half that on diets containing arginine-supplemented 20 per cent common casein. It would appear that diets containing completely hydrolyzed protein are definitely inferior to diets containing un-hydrolyzed protein.

The authors are indebted to the Works Progress Administration for the services of caretakers for the animals.

#### SUMMARY

1. Tryptophane, histidine, and arginine were found each to be essential for the chick.

2. Ornithine, urea, or ornithine plus urea was not able to replace arginine in the diet.

3. 20 per cent casein in the diet did not supply an adequate amount of arginine for the normal growth of the chick. Supplementation of a diet containing 30 per cent casein with arginine did not increase the rate of growth.

4. The rate of growth on the most favorably supplemented acid-hydrolyzed casein diets was inferior to that on diets containing unhydrolyzed casein.

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# A NEW METHOD FOR DETERMINATION OF IODINE IN FIVE CUBIC CENTIMETERS OF BLOOD OR OTHER BIOLOGICAL MATERIAL\*

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Sufficient material to contain at least 0.2 microgram of iodine is weighed or measured into a  $\frac{3}{8}$  inch Visking sausage casing,<sup>1</sup> about  $\frac{1}{2}$  inch per cc. and 1 inch to tie at each end being allowed. When blood is used, a 5 cc. sample (containing 0.015 cc. of 30 per cent potassium oxalate) is pipetted into  $4\frac{1}{2}$  inches of casing which has been moistened at one end, tied off, and the string attached to a frame; the remaining end is tied off, stretched, tied to the other side of the frame, and dried for about 60 minutes in an air bath at 100°. The drying should not be complete, as this causes brittleness; furthermore, the water vapor from the sample aids the CO<sub>2</sub> in driving out the air and thus prevents the sample from burning inside the stoking device. The excess casing at each end is cut off.

The combustion equipment (Fig. 1) includes a platinum combustion tube with brick furnace, a platinum-tipped (Fig. 2) screw feed stoking device, and a three disk absorber. The combustion tube is 225 mm. long and tapers from a diameter of 14 mm. to 10 mm. Sheet platinum, 0.1 mm. thick, is used in the construction of the tube, and the seam is rolled and welded to prevent leakage. A

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<sup>1</sup> From the Visking Corporation, Union Stock Yards, Chicago.



removable platinum disk punched full of needle holes, near the center of the tube, serves to promote complete combustion of gases. A furnace equipped with three or four Fisher burners

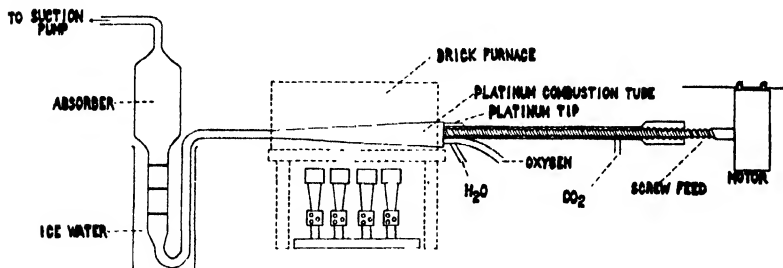


FIG. 1. Combustion train. The synchron motor is suspended on a steel knitting needle so that (as it turns the screw) it pulls itself along. The knitting needle must be supported at both ends.

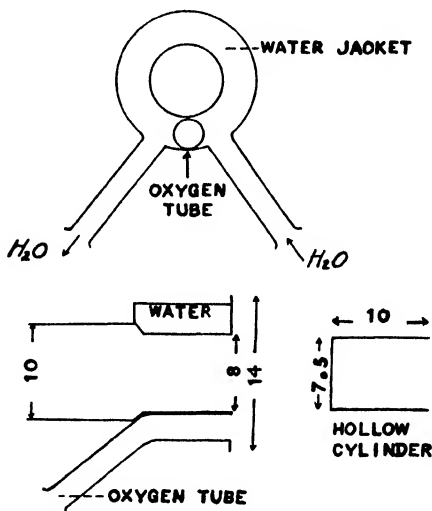


FIG. 2. Removable platinum tip to the screw feed stoking device in cross and longitudinal section. Dimensions in all figures are in mm.

heats the tube to bright redness. The stoking tube has a side inlet for  $\text{CO}_2$  to drive out the air and carries a screw feed (twenty threads to the inch) driven by a synchronous motor<sup>2</sup> (supported

<sup>2</sup> Synchron (counter-clockwise), made by Hansen Manufacturing Company, Princeton, Indiana.

by a knitting needle) 1 revolution per minute. In the absorber (Fig. 3) three sintered disks (made of pulverized Pyrex glass that pass through a 100 mesh sieve and freed from finer particles by sedimentation in water) are employed to bring the gas stream intimately in contact with the absorbent.

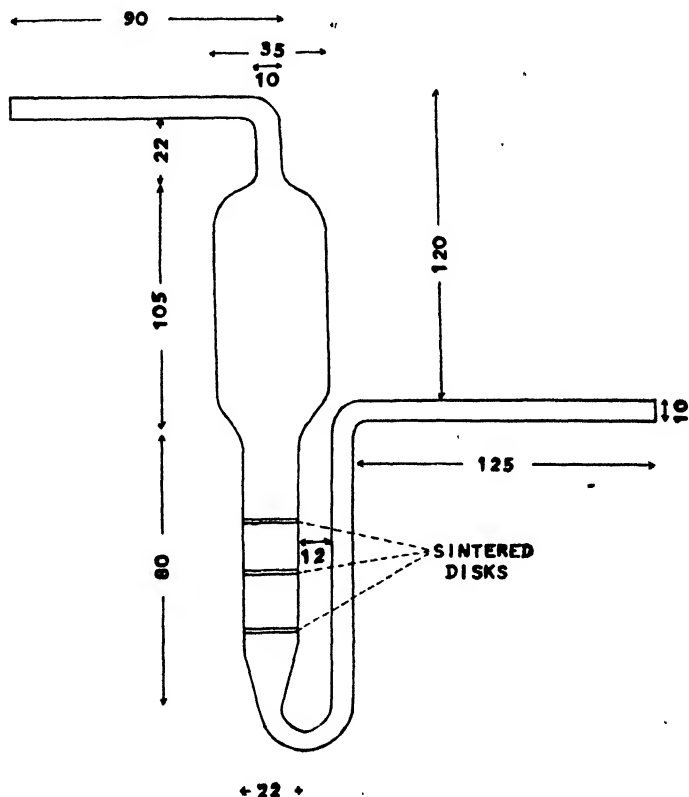


FIG. 3. Pyrex absorber

To make an analysis, the combustion tube is supported in the furnace and the small end is fitted into the absorber (immersed in melting ice) which has been charged with 10 cc. of water containing 50 mg. of sodium carbonate to make it alkaline and 50 mg. of sodium azide to reduce iodine and destroy nitrite. The

aspirator is turned on to produce 75 mm. of suction, which is enough to keep some solution above each of the three sintered disks. The sample is inserted in the stoker, which is joined to the platinum tip by means of a short piece of rubber tubing, and a very slow stream of  $\text{CO}_2$  admitted by a side inlet. The platinum tip is fitted into the large end of the combustion tube, the cooling water is started, and the oxygen tube in the tip is connected through a water manometer and a flowmeter to a cylinder of oxygen. About 120 cc. of oxygen per minute are introduced, and sufficient vacuum is applied by the water aspirator to the exit tube of the absorber to yield a pressure in the combustion tube equal to or very slightly below atmospheric pressure. The suction is made constant by a mercury regulator.

The furnace burners are lighted and the combustion tube brought to a bright red heat. The stoking motor is started and the sample burned (1 hour and 20 minutes are required for 5 cc. of blood). When the platinum plug in the stoker has been pushed past the mouth of the tip, the rubber tube is pushed back and the body of the stoking tube removed. The water flow is reduced until only steam emerges for 5 minutes. After the water and oxygen tubes are disconnected, the tip is allowed to heat for 5 minutes to distil any tarry matter into the combustion tube, where it is burned.

After the gas is turned off and the suction disconnected, the apparatus is disconnected and the contents of the absorber quantitatively transferred to the still, illustrated in Fig. 4, through a funnel, by rinsing once with 2 cc. of 0.1 N NaOH and three times with 2 cc. of water. The combustion tube is washed with 2 cc. of 0.1 N NaOH and a little water, followed by more water, the washings being added to the still contents. The tip is washed with a few drops of the NaOH and water from a wash bottle with an opening as small as a fine needle. All but about 1 cc. of the still contents is distilled off, the asbestos shield being used to prevent drying of the liquid at the sides and with just enough air, introduced through the filling tube, to prevent bumping. The short auxiliary filling tube is closed by rubber tubing and pinch-clamp. Bromine vapor is blown from the brominator (Fig. 5) into 1 cc. of water in a short 10 cc. test-tube (made by cutting in half a 15 × 150 mm. test-tube) to the point of saturation. The tube is placed

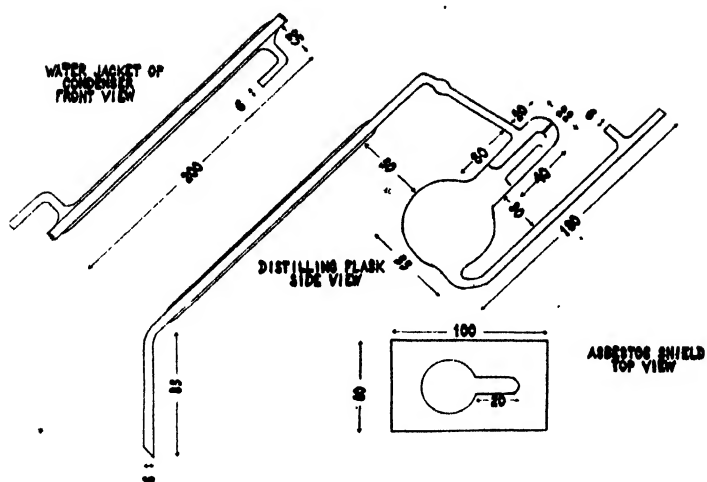
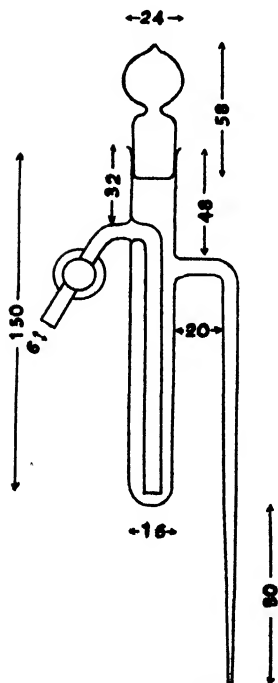


FIG. 4. Pyrex microstill



**FIG. 5. Brominator**



**Fig. 6. Microburette**

so that the tip of the condenser from the still nearly touches the surface of the bromine water. To the still contents are added 3 cc. of 6 N  $\text{H}_2\text{SO}_4$  by means of the auxiliary introduction tube. The air stream is continued to prevent bumping while the still contents are brought to the boiling point (with samples containing no iron, 0.2 cc. of 4 per cent ferric chloride or sulfate is added). While the receiver is lowered gradually to keep the condenser tip just above the liquid, the still contents are gradually distilled; a flame is applied to the trap and exit tube to keep them free from condensate. When  $\text{SO}_3$  fumes appear in the still, distillation is continued for 4 minutes with a 5 mm. flame (as in micro-Kjeldahl digestion); the condenser tip is washed down with a little water.

To the receiver content is added enough water to bring the level to 1 cm. from the top of the tube. The tube is suspended in a boiling water bath, and air is bubbled through a capillary into the liquid as rapidly as possible without loss of liquid. The stream should be slow enough to allow the large bubbles which completely span the tube to break before reaching the mouth of the tube. After the bromine color disappears aeration is continued 5 minutes more and the tube is cooled in melting ice.

Titration is made immediately as follows: To the tube, which now contains iodine as iodic acid, 1 cc. of freshly prepared KI solution (12.5 mg.) is added and the volume brought to 10 cc. The 0.001 N  $\text{Na}_2\text{S}_2\text{O}_3$  used in titration is prepared by dilution of a 0.1 N solution, 0.1 gm. of  $\text{Na}_2\text{CO}_3$  being added per 100 cc. of dilute solution to lend stability. The burette used is pictured in Fig. 6. It is constructed from a 0.1 cc. pipette graduated in thousandths. The volume between the stop-cock and the tip of the braking capillary at the top should be quite small, and the tip of the pipette should be well constricted in a long capillary which is subsequently coated with paraffin. The iodine tube is clamped to a ring-stand and a high speed stirrer inserted, which, nevertheless, does not rotate so rapidly that air is entrained. The paraffined tip of the burette is inserted just below the surface of the solution, and the concentration cell in Fig. 7 is lowered into the vessel.

The concentration cell is connected by a single pole, double throw switch, *S*, to a 1 microfarad condenser, *C*, and by throwing the switch to the opposite position, the condenser is discharged

through a high sensitivity, moving coil galvanometer,  $G$ , whose deflection is proportional to the charge on the condenser and hence to the E.M.F. of the concentration cell. The resistance,  $R$ , is in the neighborhood of 10,000 ohms, but should be adjusted to the galvanometer used so that after maximum deflection of the latter the coil returns to zero fairly rapidly. (For a low resistance galvanometer, it may be necessary to use a higher value condenser.)

The iodine solution is drawn up into the concentration cell about ten times to insure that the same concentration prevails inside as outside and verified by noting the zero potential. The level inside the cell should always be above the point of sealing in the platinum wire. A standard increment (0.005 cc.) of 0.001  $N$   $Na_2S_2O_3$  is introduced, the cell connected with the condenser,

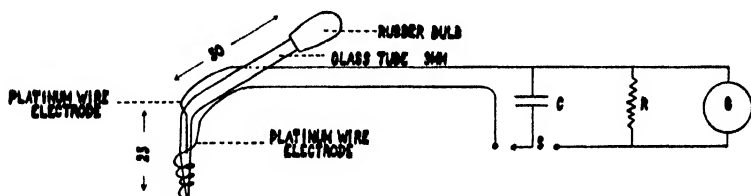


FIG. 7. Microconcentration cell and galvanometer diagram for electro-metric titration.

and 15 seconds allowed for thorough agitation and mixing of the solution and charging of the condenser. Then the condenser is discharged through the galvanometer, and the deflection is noted. The cycle is repeated until the galvanometer deflections have passed through a maximum and have decreased somewhat. The deflections are plotted against the volume of thiosulfate, with mid-points of the volume increments as plotting points; that is, 0.0025, 0.0075, and so on. The point at which the curve is at a maximum marks the end-point. In practise, it is convenient to draw straight lines through two points on each side of the maximum and discover the maximum by extrapolation of the lines until they intersect. (After the first thiosulfate titration the solution should be titrated with 0.1  $N$   $NaOH$ , phenolphthalein being used to indicate how much acid should be added to the standard.) The thiosulfate should be standardized by titration in the same

manner against pure  $\text{KIO}_3$ ; it is acidified with 5 cc. of the 0.1  $\text{N}$   $\text{H}_2\text{SO}_4$  (or the acidity of the unknown) and after addition of KI is brought to 10 cc. volume as before (the KI blank being subtracted).

There are at least three sources of substances that may augment the amount of iodine in this analysis: (1) iodide in the reagents used to absorb the iodine in the combustion and liberate it during distillation, (2) bromine remaining after aeration, and (3) iodate or free iodine in the KI used in titration. Products from reactions reduce the iodate after the bromine is blown out unless titration is made immediately.

To run a blank,  $3\frac{1}{2}$  inches of casing and 0.015 cc. of 30 per cent potassium oxalate are burned in the tube and the analysis completed as usual. This blank should not exceed about 0.1 microgram and should be subtracted from the unknown. From the iodate standard subtract the KI reagent blank (below). In the determination of blanks, increments of 0.0025 cc. of the  $\text{Na}_2\text{S}_2\text{O}_3$  should be used.

#### *Reagents—*

Distilled water. All water used should be redistilled in a non-metal still, as metals act as oxidation catalysts.

Standard  $\text{KIO}_3$ . 1.7835 gm. of recrystallized  $\text{KIO}_3$ , dried for 1 hour at  $130^\circ$ , are dissolved to make 1 liter of solution. This contains about 1058 micrograms of iodine per cc. It should be diluted to 1000 volumes.

Standard KI. Recrystallized KI dried at  $110^\circ$ ; 1.308 gm. per liter. 1 cc. of this diluted to 1 liter contains 1 microgram of iodine per cc.

Standard 0.1  $\text{N}$   $\text{Na}_2\text{S}_2\text{O}_3$ . 24.85 gm. of the pure crystalline salt and 0.1 gm. of  $\text{Na}_2\text{CO}_3$  are dissolved to make 1 liter of solution. It should be diluted to 0.001  $\text{N}$  before use, 0.1 gm. of  $\text{Na}_2\text{CO}_3$  added per 100 cc. of diluted solution, and the mixture standardized against  $\text{KIO}_3$ .

0.1  $\text{N}$   $\text{H}_2\text{SO}_4$ . 0.25 cc. of pure concentrated  $\text{H}_2\text{SO}_4$  is diluted to 90 cc.

6  $\text{N}$   $\text{H}_2\text{SO}_4$ . 40 cc. of concentrated acid per 240 cc. of solution.

KI. A solution of recrystallized KI containing 12.5 mg. per cc. is made up just before use. A blank may be run on 8 or 10 cc. 1 cc. should not contain more than 0.02 microgram of iodine.

Bromine. The purest bromine available is purified by washing three times with water ( $\text{I}_2$  dissolves in water to form iodate).

1 cc. of bromine water plus 5 cc. of 0.1 N  $\text{H}_2\text{SO}_4$  made up to 9 cc. is about the color of 0.07 per cent dichromate solution, and if aerated until the color disappears and 5 minutes more at 100°, it should not require more than 0.05 microgram of iodine for titration.

4 per cent  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . 4 gm. of crystalline, hydrated  $\text{FeCl}_3$  are dissolved in 100 cc. of water plus 2 cc. of 6 N  $\text{H}_2\text{SO}_4$ . The solution is evaporated nearly to dryness over a free flame, 100 cc. of water are added, and the operation repeated. Upon dilution to 100 cc., the solution is filtered if necessary (4 per cent ferric sulfate may be substituted if heat is used to get it into solution).

TABLE I  
*Iodine Recovered from Blood*

The original 5 cc. of blood taken for each experiment contained 0.5 microgram of iodine.

Iodine added	Total	Amount recovered	Per cent recovered	Average per cent recovered
<i>micrograms</i>	<i>micrograms</i>	<i>micrograms</i>		
5	5.5	4.81	88	
5	5.5	4.91	89	
5	5.5	5.33	97	
5	5.5	5.51	100	94
1	1.5	1.27	85	
1	1.5	1.37	92	
1	1.5	1.39	93	90
1	1.5	1.40	93.4	
1	1.5	1.43	95.4	
1	1.5	1.45	96.7	95

Sodium azide (Eastman's or Kahlbaum's). 100 mg. in 100 cc. of  $\text{H}_2\text{O}$ .

0.1 N NaOH. 0.4 gm. (c.p.) of NaOH per 100 cc. of solution, in a paraffin-lined bottle. If, as usually is the case, the NaOH contains some carbonate, 0.5 gm. is used to insure a sufficient alkalinity for alkalizing the still contents.

NaOH for titrating is dissolved in an equal weight of water and the carbonate settled; it is then diluted and titrated with standard acid and made 0.1 N. Either of the above solutions may be used in cleaning the electrodes.

Repeated determinations on beef blood gave 0.47, 0.54, 0.51,



and 0.48 micrograms of iodine in 5 cc., the average being 0.5 microgram. This blood was used in adding known quantities, as shown in Table I.

Besides the work on beef blood, the iodine has been determined in 60 samples of human blood (patients), the average value being 0.5 microgram in 5 cc.

#### DISCUSSION

The senior author has been engaged in iodine microanalysis since 1922 (1) and was the first to make closed combustions on biological materials, both in a bomb (2) and a combustion tube (silica) (3). This was improved by the screw feed stoking device (4) and adapted to analysis of 10 cc. of blood (5).

In the present method as announced (6) a platinum combustion tube is used and an electrometric method has been modified for titration of small volumes.

The differential method (7) with the pipette electrode (8), but reduced in size, gave satisfactory results, provided the condenser and switching arrangement described above were employed. The platinum wire must not be too small and must be heated to a white heat after being bent into shape.

The KI solution should be made up fresh, as a sample that had stood 3 days increased its "free iodine" content from about 0.01 microgram per cc. to 0.074 microgram per cc. Since the total blank on all the reagents should not be more than about 0.1 microgram and 1 cc. of KI solution is used in an analysis, it is imperative to use fresh KI solution.

The burette used in titration was 0.1 cc., graduated in thousandths and with a stop-cock and air capillary at the top (9). Uniform increments should be added, for unequal increments near the end-point necessitate an additional calculation. It was found that 0.005 cc. increments gave more reproducible results than 0.0025 cc. increments. In titration of very small quantities of iodine, the smaller increments are imperative.

With such a small burette, the  $\text{Na}_2\text{S}_2\text{O}_3$  cannot be added to the iodine solution fast enough to endanger precipitation of sulfur unless the acidity is very high, but it may be best to have the acidity of the standard equal to that of the unknown. Since in analysis of 5 cc. of blood, HCl (from blood chlorides) equivalent to

about 3.5 cc. and  $\text{SO}_2$  equivalent to about 1.5 cc. of 0.1 N  $\text{H}_2\text{SO}_4$  are distilled, the effect of 5 cc. of 0.1 N  $\text{H}_2\text{SO}_4$  on the titration was studied and the type of curve found to be excellent.

Iodic acid is perfectly stable at  $100^\circ$  for 30 minutes, as shown by the following experiments. 0.5 microgram of iodine as iodate was added to each of four tubes. To these were added 1, 5, 5, and 7.5 cc. of 0.1 N  $\text{H}_2\text{SO}_4$ . Tube II was used as standard and Tubes I, III, and IV were made up to 9 cc. and aerated at  $100^\circ$  for 30 minutes. Tube I titrated 0.5 microgram; Tube III, 0.5 microgram; and Tube IV, 0.504 microgram.

In order to determine the "bromine blank," two series of four titrations each were made on acidified bromine water alone. In the first series the total bromine added was constant, but the aeration of the first was so great as to break bubbles at the top of the tube, and the others progressively decreased. The titrations were 0.0045, 0.0048, 0.0045, and 0.005 cc. of thiosulfate. In the second series the aeration was constant, but the quantity of bromine water was one-ninth, two-ninths, three-ninths, and four-ninths of the amount required to match 0.07 per cent  $\text{K}_2\text{Cr}_2\text{O}_7$  solution. The titers were 0.0044, 0.0042, 0.0045, and 0.0042. It seems possible to make the bromine blank constant by a uniform technique, although our experiments fail to determine whether this blank is due to iodine in the bromine or bromine in the titration. If the aeration is not performed in a hood, it is necessary to open the windows before titrating. The "bromine blank" is not due to iodine in the 0.1 N  $\text{H}_2\text{SO}_4$ , since a series of three tubes with 1, 2, and 5 cc. of this acid titrated 0.004, 0.0045, and 0.0045 cc. of thiosulfate.

When more than 5 micrograms of iodine (as iodate) are to be titrated, the electrodes may be kept out of the solution until all but about 1 microgram of the iodine has been reduced; otherwise, the high iodine concentration may polarize the electrodes. 0.1 N  $\text{NaOH}$  may be used to clean them even during the titration, if the end-point has not been approached, for the galvanometer deflections observed before cleaning the electrodes cannot be used in plotting the end-point.

In case the amount of iodine recovered from the burning of a blood sample is less than twice that found in the blank, a duplicate should be burned or a larger sample analyzed. If not enough

oxygen is used or if the tip is not heated long enough or is heated too rapidly after introducing the last of the sample into the combustion tube, traces of colored material other than iron may reach the receiver. If they are not completely destroyed by boiling the  $\text{H}_2\text{SO}_4$  in the still 4 minutes after all the water has been driven off, the results will be too low and another sample must be burned.

#### SUMMARY

A microcombustion tube and distillation method for isolating the iodine in 5 gm. of biological material (containing more than 0.2 microgram) is described.

An electrometric titration method is so refined that it seems to be 1000 times as sensitive as the starch-iodide method.

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## STUDIES IN AMINO ACID METABOLISM

### IV. METABOLISM OF *dl*-PHENYLALANINE AND *dl*-TYROSINE IN THE NORMAL RAT\*

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The metabolism of the aromatic amino acids, phenylalanine and tyrosine, has excited the interest of nutritionists for many years. Until recently it has been generally assumed that these acids follow the same pathway in metabolism after the preliminary oxidation of phenylalanine to tyrosine (1). The fact that both aromatic amino acids are precursors of homogentisic acid in the condition of alcaptonuria (2) as well as the evidence that both yield acetone bodies when perfused through a surviving liver (3) has given additional support to the identity of their intermediary metabolism. Also neither tyrosine (4) nor phenylalanine (5) has been shown to be capable of producing sugar in the phlorhizinized dog.

On the other hand, Shambaugh, Lewis, and Tourtellotte (6) have concluded that their data do not support such a theory. This is based upon the finding of a significant amount of phenylpyruvic acid in the urine of rabbits receiving phenylalanine. No excretion of *p*-hydroxyphenylpyruvic acid followed the ingestion of tyrosine. More recently, Womack and Rose (7) have shown that phenylalanine is essential in nutrition and that tyrosine cannot replace it. Therefore, it seems that the theory of a single metabolic pathway may not be as certain as it was at one time thought to be. In the present study, the comparative glycogenic and ketolytic activities of these amino acids have been followed;

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further evidence is here presented, showing the dissimilarity in the metabolism of these acids.

#### EXPERIMENTAL

The procedures used previously (8) were employed in the present study. In the first series of tests glycogen was determined at definite intervals during which maximum absorption of either phenylalanine, tyrosine, or alanine was allowed to occur. In the second group of experiments the effect of the amino acids on the ketonuria produced by sodium butyrate was followed. In both cases comparisons were made simultaneously with fasting controls which received gum tragacanth. *dl*-Alanine has been shown to be an excellent source of glycogen (8) and was used for comparison.

For the glycogen studies female rats ranging from 100 to 170 gm. were used. Owing to the insolubility of phenylalanine and tyrosine, these acids were fed in a 10 per cent suspension in a 1.75 per cent gum tragacanth medium. The material was ground to a very fine powder, the tragacanth suspension added, and the whole shaken vigorously. By this method uniform concentrations of the amino acids were found in different samples which were analyzed. An accuracy of 3 to 4 per cent is easily realized with samples containing 75 to 100 mg. of the acid. After a preliminary 48 hour fast a larger amount of the suspension was fed to the rats by stomach tube than could be absorbed during a definite period, and groups of animals were killed at the end of 3, 6, 9, and 12 hours. Amytal was used as an anesthetic. The livers were removed and analyzed for glycogen, according to the Good, Kramer, and Somogyi technique (9); the gastrointestinal tract was removed, minced, the protein precipitated with trichloroacetic acid, and the whole filtered. Analyses for amino nitrogen by the Van Slyke procedure were made to determine rates of absorption. These rates will be reported in a later publication. Filter paper was placed in the cages prior to feeding of the amino acids to supply roughage. This does not change the rate of absorption of the amino acids and it greatly lessens the chance of diarrhea (10).

To determine the metabolism in regard to acetone body production or destruction, the acids were fed alone in one series and in a second group of experiments were administered to animals which were receiving sodium butyrate. Urine collections were made

every 24 hours, as described previously (11). The Van Slyke technique was used for total acetone body determination and the Kjeldahl method for total nitrogen. The procedure of Briggs (12) was employed to determine homogentisic acid. The amino acids used in this study were prepared by one of us (M. S. D.), and in all cases only products of highest purity were used.

### Results

Four control groups of seven females in each group were used in the glycogen studies. The values for the individual groups are

TABLE I

*Glycogen Content of Livers of Female Rats after Being Fed Various Amino Acids Suspended in 1.75 Per Cent Gum Tragacanth Medium (Maximum Absorption at All Times)*

Time after feeding	Glycogen				Ratio, mean difference to probable error of mean difference					
	I. Control	II. <i>dl</i> - Tyro- sine	III. <i>dl</i> - Phenyl- alanine	IV. <i>dl</i> -Ala- nine	I:II	I:III	I:IV	II:III	II:IV	III:IV
hrs.	per cent	per cent	per cent	per cent						
3	0.09 (7)	0.13 (7)	0.17 (7)	0.85 (6)	2.35	9.17	6.70	2.30	6.43	6.01
6	0.09 (7)	0.10 (8)	0.28 (8)	1.56 (7)	1.22	8.56	31.6	7.72	31.6	25.2
9	0.06 (7)	0.12 (6)	0.71 (7)	2.59 (7)	4.96	5.90	9.53	5.36	9.35	6.41
12	0.06 (7)	0.29 (6)	0.75 (7)	2.33 (7)	5.43	12.72	14.70	6.70	12.72	9.57

Figures in parentheses refer to the number of experiments.

as follows: 3 hour control, 0.09 per cent; 6 hour, 0.09; 9 hour, 0.06; and 12 hour, 0.06 per cent. In the twenty-eight experiments the values ranged from 0.02 to 0.14 per cent. These results, as well as those obtained after feeding phenylalanine and tyrosine, are recorded in Table I.

The liver glycogen values following administration of phenylalanine were 0.17, 0.28, 0.71, and 0.75 per cent, respectively, for the 3, 6, 9, and 12 hour groups, and for tyrosine, 0.13, 0.10, 0.12, and 0.29 per cent for the same time intervals. The comparative levels of liver glycogen after *dl*-alanine were 0.85, 1.56, 2.59, and

2.33 per cent. Statistical analysis of all these data is reported in Table I.

The influence of the administration of the amino acids on the acetonuria developed in male rats is reported in Tables II and III.

TABLE II

*Excretion of Acetone Bodies by Male Rats*

The experimental animals received either 11.5 gm. of *dl*-alanine, 25.0 gm. of *dl*-tyrosine, or 24.0 gm. of *dl*-phenylalanine per sq.m. of body surface per day as compared with the controls which received gum tragacanth only. The acids were suspended in a 1.75 per cent gum tragacanth medium. All animals received 15.0 gm. (as acetone) of sodium butyrate per sq.m. per day. Four rats were included in each group.

Material fed	Acetone body excretion			
	Gm. per sq. m.			Average for 3 days, mg. per 100 gm. rat
	2nd day	3rd day	4th day	
Control . . . . .	3.87	4.28	4.84	60.3
<i>dl</i> -Alanine . . . . .	1.35	0.79	1.85	18.9
<i>dl</i> -Tyrosine* . . . . .	3.07	4.35	5.49	60.5
<i>dl</i> -Phenylalanine . . . . .	2.53	1.78	2.22	30.4

\* Three experiments only on the last day.

TABLE III

*Statistical Analysis of Acetone Body Production by Male Rats Receiving Sodium Butyrate Plus Various Amino Acids Suspended in 1.75 Per Cent Gum Tragacanth*

Results are expressed in gm. per sq.m. of surface area.

Material fed	No. of ex- periments	Acetone body excretion		Mean	Prob- able error of mean	Ratio, mean difference to probable error of mean difference			
		Mini- mum	Maxi- mum			I	II	III	IV
I. Control . . . . .	12	2.48	7.28	4.33	0.296		9.17	0.24	7.25
II. <i>dl</i> -Alanine . . . . .	15	0.08	2.61	1.33	0.147	9.17		7.34	3.25
III. <i>dl</i> -Tyrosine . . . . .	14	0.81	7.48	4.22	0.365	0.24	7.34		5.46
IV. <i>dl</i> -Phenylalanine . . . . .	15	0.59	3.89	2.07	0.139	7.25	3.25	5.46	

The experiments were continued for 4 days. The 1st day the animals received only sodium butyrate, no analyses being made. During the following 3 days separate analyses were made daily for total acetone bodies and total nitrogen.

The acetone body excretion, expressed in gm. per sq. m. of surface area, for the 2nd, 3rd, and 4th days respectively, is as follows: for the controls, 3.87, 4.28, and 4.84; the alanine group, 1.35, 0.79, 1.85; the tyrosine group, 3.07, 4.35, and 5.49; while the rats receiving phenylalanine excreted 2.53, 1.78, and 2.22 gm. of acetone bodies per sq. m. per day. For comparison the final column of Table II gives the mean of all experiments for 3 days, expressed in mg. per 100 gm. of rat. These results, of course, show the same trend.

TABLE IV

*Excretion of Acetone Bodies, Urinary Nitrogen, and Homogentisic Acid by Male Rats*

The experimental animals received either 31.20 gm. of *dl*-tyrosine or 28.60 gm. of *dl*-phenylalanine per sq. m. of body surface per day as compared with the controls which received gum tragacanth only. All acids were fed in a 10 per cent suspension in a 1.75 per cent gum tragacanth medium.

Material fed	Acetone body excretion, gm. per sq. m.			Total nitrogen excretion, gm. per sq. m.			Homogentisic acid			
	1st day	2nd day	3rd day	1st day	2nd day	3rd day	Gm. per sq. m.			Mg. per 100 gm. for 3 days
<i>dl</i> -Tyrosine . . . . .	0.05 (6)	0.03 (6)	0.08 (6)	4.39 (6)	3.80 (6)	3.79 (6)	0.00	0.00	0.00	0.00 (18)
<i>dl</i> -Phenylalanine . . . . .	0.07 (6)	0.02 (6)	0.07 (5)	5.58 (6)	5.26 (6)	5.13 (5)	0.52 (6)	0.83 (6)	2.56 (5)	25.3 (17)
Fasting control . . . . .	0.03 (8)	0.02 (8)	0.03 (7)	4.48 (8)	3.88 (8)	3.78 (7)	0.00	0.00	0.00	0.00 (23)

Figures in parentheses refer to the number of experiments.

In Table IV the effect of feeding isomolecular amounts of *dl*-tyrosine and *dl*-phenylalanine alone (no sodium butyrate) is shown. Homogentisic acid was excreted by the animals receiving phenylalanine, the values for the 3 days being 0.52, 0.83, and 2.56 gm. per sq. m. The urinary nitrogen for these animals is also reported, and for the 3 days the levels were 4.39, 3.80, and 3.79 gm. per sq. m. for the tyrosine group, while the animals receiving phenylalanine excreted 5.58, 5.26, and 5.13 gm.; the corresponding values for the control group were 4.48, 3.88, and 3.78 gm. No appreciable ketonuria could be demonstrated in any experiments.



## DISCUSSION

From the results reported in Table I it would seem that phenylalanine must be regarded as a precursor of glycogen in the normal intact animal.

Even in the 3 hour group definite glycogen formation followed the administration of phenylalanine, although the level is only 0.08 per cent above the control. This may be traced to the extremely constant value found in both the control and experimental animals. Young females, 85 to 100 days old, were chosen for this work, because we have found that at this age a 48 hour fast reduces the liver glycogen to a very low constant level. The value of liver glycogen for the 6, 9, and 12 hour periods is also found to be significantly higher than that of the animals receiving tyrosine, or of the control rats. In the animals receiving tyrosine there seems to be a small amount of glycogen deposited in the liver in the 9 and 12 hour groups. Actually these values are found to be statistically significant, although the increase is only slight. In confirmation of our earlier results high glycogen levels were consistently found after the administration of *dl*-alanine.

In unpublished observations of Lewis (13) essentially similar results have been found; namely, a significant glycogen formation after either *l*- or *dl*-phenylalanine, while after *l*-tyrosine only small amounts of this polysaccharide were formed.

Confirmatory evidence of the fate of these two amino acids in metabolism is furnished when their effect on an artificially induced ketosis is noted. Acetone body production in the tyrosine group is almost identical with that found in the control group, thus lending support to the thesis that this acid is a very poor glyco-genic agent. These data do show that tyrosine does not contribute to acetone body production; otherwise the acetonuria should be increased. Such results have been found after feeding an amino acid like leucine which does have as its fate in metabolism acetone body formation (14).

After phenylalanine administration there is an unmistakable decrease in the acetonuria. The demonstration of the ketolytic effect is strong evidence for concluding that sugar formation is one fate of this compound in metabolism. Statistical evaluation of these results has been made from the total number of experi-

ments over the 3 day experimental period. These values are given in Table III and show phenylalanine to cause significantly lower results than are found in either the tyrosine or the control group. Again alanine is superior to either of the aromatic amino acids.

When the urines were subjected to the cupric hydroxide precipitation preliminary to determination of acetone bodies, it was noted that those from the animals given phenylalanine caused the formation of black cuprous oxide. On investigation this material proved to be homogentisic acid. A maximum of 130 mg. was excreted on the 3rd day. The average daily values in gm. per sq. m. per day are given in Table IV. It is significant that blank values were invariably found after tyrosine as well as in the control urines.

The differences in response of the two aromatic amino acids cannot be traced to the non-absorption of tyrosine. In the first place the amounts of the amino acids which were fed (31.2 gm. per sq. m. per day for tyrosine and 28.60 gm. for phenylalanine) are well within the absorption ranges we have found for these two acids when fed in a similar suspension and we do not believe this difference in behavior could be traced to lack of absorption. Moreover, since we were feeding an average of 9 cc. of the suspension each day without encountering any diarrhea, it is impossible that the material remained in the gastrointestinal tract. Despite the fact that tyrosine must have been absorbed, there was no increase in excretion of urinary nitrogen over the control level, although significantly higher values were found in the urine after phenylalanine. This failure of tyrosine to show increased urinary nitrogen is in line with the findings of Folin and Denis (15) who were unable to show an increase in non-protein and urea nitrogen in the blood after injection of tyrosine into the intestine of a cat. Similar results are reported by Kiech and Luck (16), using rats as experimental animals. King and Rapport (17) were unable to discover the fate of tyrosine after intravenous injection of 5 gm. of the substance in dogs, neither the acid nor the nitrogen from the acid being excreted. Since it seems improbable that lack of absorption is the explanation of these observations, further work must be done before answering this question.

## SUMMARY

1. *dl*-Phenylalanine, when fed to fasting rats, causes a definite deposition of glycogen.

2. Glycogen determination after *dl*-tyrosine gave inconclusive evidence concerning its fate in this respect, although there appeared to be slight deposition of this polysaccharide.

3. *dl*-Phenylalanine when fed at a level of 28.60 gm. per sq. m. evoked an excretion of homogentisic acid. When *dl*-tyrosine was fed in an isomolecular amount, no trace of homogentisic acid appeared in the urine.

4. No evidence was found of acetone body formation after either acid.

5. *dl*-Phenylalanine but not *dl*-tyrosine caused a decrease in the acetonuria produced by the feeding of sodium butyrate.

6. Nitrogen excretion following administration of *dl*-tyrosine is at the same level as in the control group, while *dl*-phenylalanine caused a significantly higher excretion.

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## A STUDY OF THE PROTEINS OF THE INACTIVE AND ACTIVE MAMMARY GLAND\*

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According to Sørensen (23), native proteins consist essentially of systems of components that differ in solubility in suitable solvents and to a minor extent also in chemical composition. A protein *in situ* is therefore a system, rather than a chemical entity. Nevertheless it might be anticipated that the mixture of components brought into solution, or peptized, by a solvent under standardized conditions would be reproducible, and comparison of preparations secured from a given tissue may thus yield information of physiological value. The present paper describes the properties of preparations obtained by a fixed procedure from bovine mammary glands at different stages of lactation, and an attempt is made to interpret the results in terms of the synthetic activity of this gland.

Although the properties of preparations of proteins secured from many gland tissues have been recorded (1, 13, 25, 14, 16), very few chemical studies of the mammary gland have been made. Laxa (15) and Gowen and Tobey (10) have reported the proximate composition of mammary glands secured from the cow, and Petersen, Palmer, and Eckles (21) have studied the fat content of similar tissues. Meigs, Blatherwick, and Cary (17) have investigated the relationship between the phosphorus of the blood and of the milk, and a similar study has been made by Blackwood

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and Stirling (2). Drummond (4) has recorded a brief analysis of a protein preparation secured from the human mammary gland.

#### EXPERIMENTAL

*Material*—The tissues used in this work<sup>1</sup> are designated as follows, in the order of their apparent activity according to previous history or inspection of the gland: heifer gland, a composite of several glands from heifers which had never been pregnant;<sup>2</sup> dry Gland A, two completely developed udders from cows in a non-lactating condition, obtained from the packing company, these glands had no milk in the ducts. Dry Gland B, this cow had become dry and had not been milked for several weeks. However, the gland contained considerable milk in the ducts. Therefore this material cannot be considered as inactive as was dry Gland A. Nearly dry gland, two glands which contained appreciable quantities of milk in the lacteal ducts; active Gland A, from a cow in moderately active lactating condition; active Gland B, from an actively lactating good milk cow; active Gland C, from the packing plant where there were no available data on milk production, but from appearance this was the most active gland studied; muscle, a piece of muscle cut from the rump of the same animal that furnished active Gland C; lymph glands, the supramammary lymph glands situated above the posterior border of the base of the mammary gland, collected from a number of animals. These glands varied in size from 1½ to 4 inches.

*Preparation of Tissues and Protein Fractions*—The active glandular portions, freed from all extraneous tissues, were washed free

<sup>1</sup> Our sincere thanks are due to Swift and Company, South St. Paul, and to the Division of Dairy Husbandry of the Minnesota Agricultural Experiment Station for their fine cooperation and assistance in providing these glands. We are particularly indebted to Professor W. E. Petersen of the Division of Dairy Husbandry for advice and assistance in classifying the glands. The entire udder was removed from the animal immediately after slaughter. The actual glandular tissue was dissociated from the fat, skin, and connective tissue and the glandular tissue prepared for extraction within as short a time as was possible following the removal of the gland.

<sup>2</sup> Because the removal of the undeveloped udder from a young carcass causes the meat to fall into a cheaper grade, it was not possible to obtain the glands from freshly killed heifers. The glands were removed from carcasses which had been in the cooler at +3° for 2 to 5 days.

from milk and ground in a mill. A part of the pasty mass secured was dehydrated with acetone and analyzed (Table I, original). The remainder was divided into two parts, one of which was extracted with 0.8 per cent hydrochloric acid, the other with 10 per cent sodium chloride as follows: The tissue was suspended in a glass jar equipped with a mechanically driven glass stirrer, and agitated with from 10 to 20 times its weight of solvent at 5° for 12 hours. The suspension was then transferred to a canvas bag, 5 × 36 inches, which was then placed circumferentially in a 12 inch, rubber-lined basket centrifuge and centrifuged at 1700 R.P.M.

The 10 per cent sodium chloride extract was filtered and dialyzed against distilled water at 0-5°. The precipitated protein was removed, dehydrated with acetone, extracted with ether, dried, and analyzed (Table I, globulin). The filtrate from the globulin was acidified with acetic acid and the protein fraction which coagulated on heating at 85° for 30 minutes was separated, again suspended in water and electrodialed, dehydrated with acetone, extracted with ether, dried, and analyzed (Table I, albumin).

The 0.8 per cent hydrochloric acid extract was treated with an exact equivalent of sodium acetate and the protein precipitated from the acetic acid solution by adding sodium chloride to 10 per cent concentration. The protein precipitate was suspended in water, dialyzed, and electrodialed. The insoluble residue was dehydrated with acetone, extracted with ether, dried, and analyzed (Table I, histone).

### Methods

Van Slyke's (26) nitrogen distribution method with minor modifications (3, 11, 22) was employed. All hydrolyses were of exactly 24 hours duration. Cystine was determined colorimetrically (7), with a picric acid filter (6). Phosphorus was determined colorimetrically (5) on the protein ash, the ashing being conducted in the presence of calcium acetate (18).

### DISCUSSION

Preliminary quantitative extractions, by the method of Gortner *et al.* (9), had shown that 0.8 per cent hydrochloric acid peptized approximately 22 per cent and 10 per cent sodium chloride pep-



Active Gland B	Original	5.50	1.26	14.70	6.2	2.7	27.0	63.1	13.8	12.9	0.13	5.2	8.7	53.0	10.1
	Albumin	0.49	0.04	15.10	6.9	1.8	28.0	62.4	17.5	11.6	0.30	2.8	13.4	59.7	2.7
	Globulin	1.50	0.61	15.40	7.1	2.2	29.9	60.7	15.2	15.2	0.07	5.1	9.6	54.2	6.5
	Histone	0.72	0.24	15.40	7.4	1.6	27.3	63.0	15.1	13.2	0.27	2.3	11.6	45.4	17.5
" C	Original	10.9	2.00	15.80	6.2	1.8	23.7	67.4	11.4	12.0	0.05	4.9	6.7	57.9	9.6
	Albumin	0.16	0.01	15.20	8.4	1.6	27.8	62.2	16.2	10.8	0.17	5.2	11.7	58.3	3.9
	Globulin	2.30	0.93	15.70	9.0	2.4	29.9	57.9	15.1	16.3	0.16	3.9	9.6	52.0	5.9
	Histone	0.90	0.29	15.40	6.9	3.1	27.0	62.6	13.7	12.8		5.5	8.7	50.0	12.6
Suprarenal	Albumin	0.50	0.04	15.20											
lymph	Globulin	1.60	0.90	16.30	8.3	2.7	31.9	55.7	17.2	14.7	0.12	5.5	11.5	50.9	4.8
	Histone	1.60	0.43	15.40	7.0	1.9	31.1	59.5	16.1	13.9	0.19	6.9	10.1	52.6	6.9
Muscle tissue	Original	5.0	0.90	16.10	7.6	1.9	30.4	60.2	16.6	15.2	0.09	3.6	11.5	56.6	3.7
	Albumin	0.48	0.02	15.90											
	Globulin	2.50	0.46	16.40	7.6	1.1	29.7	61.6	17.4	12.0	0.06	4.8	12.8	60.7	0.9
	Histone	0.66	0.06	15.50	7.1	1.5	30.2	61.7	18.7	12.6	0.14	3.1	14.3	59.3	2.4

\* Ash- and moisture-free basis.



tized approximately 28 per cent of the total nitrogen of the glandular tissues. Under our experimental conditions essential equilibrium was reached between tissue and solution in approximately 3 hours. The first sodium chloride extract peptized 4 times as much protein as did a second extract. The first hydrochloric acid extract peptized 7 times as much nitrogen as did the second extract.

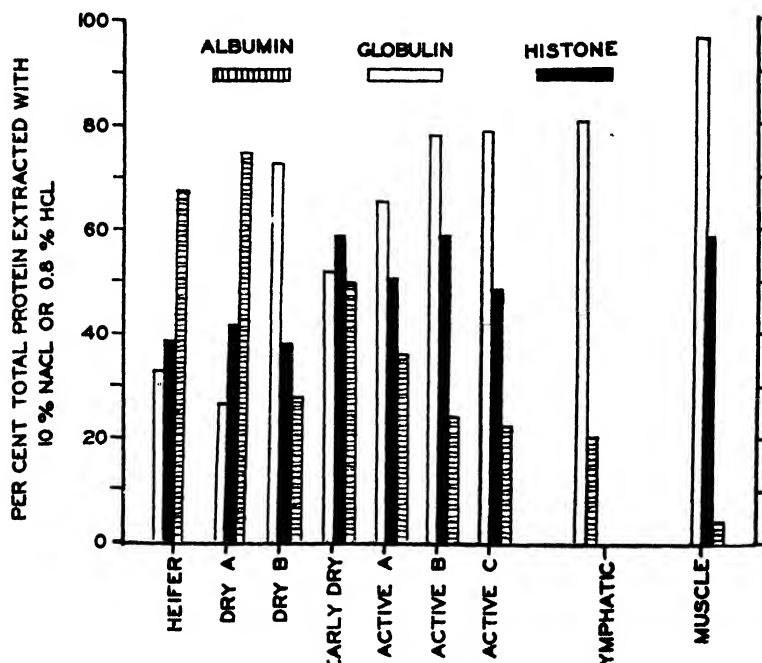


FIG. 1. Showing the changes in the protein fractions isolated from the various glandular tissues with changes in glandular activity.

There was no correlation between the total amount of protein peptized and the apparent activity of the glands. However, when the various fractions are considered there are some definite trends. These are shown in Fig. 1. The albumin to globulin ratio is reversed as we pass from the inactive to the active mammary glands. Dry Gland B is an exception to this generalization. On dissection this gland appeared to be quite active and milk was found in the

ducts. Apparently it was more active than the preliminary diagnosis of the animal had indicated. The lymphatics, presumably very active glands, have an albumin to globulin ratio simulating that of the active mammary glands.

The histone fraction tends to increase with glandular activity. Here the nearly dry gland is somewhat out of line and shows peptization behavior more nearly characteristic of the more active tissues.

The separation of the globulin and albumin from the sodium chloride extract is an arbitrary procedure (8, 9, 24) and the relative proportion of the two fractions depends upon the technique which is employed. All of our samples were treated as nearly as possible alike. Moulton (19) fractionated a water extract of beef muscle into globulin and albumin by salting-out the former with half saturated zinc sulfate. When the albumin was calculated by difference, a 1:1 ratio of globulin to albumin was obtained, but when the albumin was determined by heat coagulation, the ratio was about 3:1. With another technique Howe (12) obtained a globulin to albumin ratio of 5:1. The dialysis technique which we used gives a ratio of 24:1.

There are no clear-cut trends in the nitrogen distribution figures which differentiate the active glandular tissue from the inactive tissue. Perhaps the increased arginine content in the globulin fraction of the active tissue is significant. The arginine content of comparable series does differ significantly in the order of globulin > histone > albumin, whereas the total basic nitrogen follows the order, globulin > albumin > histone. No evidence is obtained from either the total protein nitrogen or the total basic nitrogen that the hydrochloric acid is extracting a true histone from either the mammary or lymph gland tissue.

The nitrogen distributions of the mammary tissue fractions are remarkably similar to those of the supramammary lymph gland fractions and the muscle tissue fractions, although the lymph gland fractions appear to have significantly higher total basic nitrogen values. Our beef muscle analyses are within the range of those reported by Moulton and Sieveking (20) except for humin and cystine.

There is a significant increase in histone phosphorus with increase in glandular activity. This is not the case for the albumin

phosphorus. All histone and albumin fractions were electro-dialyzed; this residual phosphorus must accordingly be regarded as an integral part of the protein fraction. Unfortunately the original and globulin fractions were not electro-dialyzed, which probably accounts for their high phosphorus contents. The lymph gland histone fraction likewise has a high phosphorus content which is not found in the muscle tissue histone. Apparently a high phosphorus content in that fraction of the glandular proteins which is extractable by dilute hydrochloric acid is indicative of glandular activity.

#### SUMMARY

Mammary gland tissue, supramammary lymph gland tissue, and muscle tissue are more completely peptized by 10 per cent sodium chloride solutions than by 0.8 per cent hydrochloric acid solutions. The hydrochloric acid extracts from active glands a larger proportion of the protein nitrogen than it does from inactive glands.

The inactive glands yield more albumin than globulin to the sodium chloride extract, but this ratio is markedly reversed for active glands. The lymph gland extract had an albumin to globulin ratio simulating that of the active mammary glands.

Nitrogen distributions of the protein fractions isolated from glands of various activities show no really significant differences which can be correlated with glandular activity. Protein fractions peptized by different solvents and exhibiting radically different physical properties show very similar nitrogen distributions.

Neither the total nitrogen nor the basic nitrogen of the protein fraction extracted by hydrochloric acid is indicative of the presence of a true histone in either the mammary gland or the supramammary lymph glands.

The phosphorus content of the different histones increases with glandular activity. The lymphatic gland histone is similar in phosphorus content to the histone of the active mammary glands. A relatively high protein phosphorus content is apparently indicative of glandular activity.

Differences between protein tissues of active and inactive glands appear to be reflected in their physical properties, such as peptization response, rather than by differences in their amino acid composition.

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## THE AMINO ACIDS OF CERTAIN MARINE ALGÆ\*

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In this communication is reported a preliminary attempt to ascertain whether any correlation exists between the nature of the chemical components of the autotrophic plants and their position in the evolutionary series. In attacking this problem, the exploration of which was suggested by Dr. J. B. Conant (1), attention has been primarily devoted to the amino acids which are present as constituents of the total proteins of certain marine algæ and must necessarily have been synthesized by the organism from inorganic materials.

Although the carbohydrates and lipids of algæ have been extensively investigated, little is known of the nitrogenous constituents beyond recognition of their protein nature (2-9). In the present study, quantitative determinations have been made of the amino acids in the following algæ: *Ulva lactuca*, a marine green alga; *Laminaria* and *Sargassum*, marine brown algæ; and *Chondrus crispus* (Irish moss), a marine red alga. For purposes of comparison, selected analyses were also carried out on *Phormidium*, a fresh water plant belonging to the blue-green group, and on a much more advanced plant, the fern *Osmunda claytoniana*.

In Tables I and II are recorded the results obtained with the various algæ, listed in the phylogenetic order based on their structure and methods of reproduction. The values reported in Table II represent the percentage distribution of nitrogen in the material extracted from the algæ by hot 90 per cent formic acid. As tests on a wide variety of proteins have in every case demonstrated

\* This report is from a dissertation submitted by Abraham Mazur in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

their complete solubility in formic acid, it is assumed that this fraction comprises all protein components. A similar procedure

TABLE I  
*Percentage of Nitrogen in Lipid-Free Algæ*

	<i>Phormidium</i>		<i>Ulva</i>		<i>Laminaria</i>	<i>Sargassum</i>	<i>Chondrus</i>	<i>Osmunda</i>
	Specimen I	Specimen II	Specimen I	Specimen II				
Total .....	2.00	2.65	2.04	1.96	0.77	1.38	1.22	1.58
Extracted by formic acid..	1.40	2.09	1.90	1.77	0.57	0.99	1.03	1.17

TABLE II  
*Percentage Distribution of Formic Acid-Soluble Nitrogen*

	<i>Phormidium</i>		<i>Ulva</i>		<i>Laminaria</i>	<i>Sargassum</i>	<i>Chondrus</i>	<i>Osmunda</i>
	Specimen I	Specimen II	Specimen I	Specimen II				
Total humin .. . . .		17.5	19.1		17.7	20.1	18.5	
Ammonia.....		7.8	5.5		7.3	8.1	6.8	
Cystine .....	0.0	0.0	1.8	2.1	3.4	2.9	1.6	0.6
"Methionine".....		2.0	0.0	0.0	0.0	0.0	0.0	1.1
Tyrosine.....	1.5	1.8	0.0	0.0	1.9	1.4	2.3	
Tryptophane.....		0.2	0.3	0.6	1.1	1.5	1.6	
Histidine.....	3.5	3.8	1.2		1.6	3.2	1.8	3.7
Arginine.....	9.2	9.2	7.5		16.1	8.0	10.2	7.6
Lysine.....	0.0	0.0	0.0	0.0	0.0	5.3	4.0	3.6
Proline.....		7.0	7.0		7.6	7.3	7.1	
Oxyproline.....		2.3	3.5		1.8	2.6	1.0	
Glycine.....		1.6	0.8		2.7	0.5	2.1	
Glutamic acid.....		4.4	7.6		7.3	2.9	8.2	
Aspartic " .....		0.9	4.1		1.9	5.9	2.5	
Leucine.....		2.1	5.2		2.5	0.3	5.3	
Phenylalanine.....		1.1	2.3		1.0	0.3	1.5	
Alanine.....		5.2	6.5		6.4	4.8	3.7	
Valine.....		6.7	5.2		5.1	6.4	2.8	
Non-amino acid N.....		2.7	2.1		3.4	6.6	3.1	
Total.....		76.1	79.7		88.8	88.1	84.1	

was published, during the progress of this investigation, by Wilkins (10).

Some nitrogen was extracted from the wet tissues during the preliminary washing with alcohol. However, this never reached 10 per cent of the total, and as there is little likelihood of its comprising amino acids not represented in the principal fraction, its nature was not investigated.

In all of the four marine algæ examined, *Ulva*, *Laminaria*, *Sargassum*, and *Chondrus*, the organic sulfur is in an alkali-labile form, and may be assigned to cystine; methionine is lacking. Tyrosine is absent in the most primitive, *Ulva*, but is present in the others. Lysine could not be detected in *Ulva* and *Laminaria*, but was found in the higher algæ, *Sargassum* and *Chondrus*.

In the fresh water alga, *Phormidium*, cystine is absent; however, the organic sulfur, if present as methionine, would account for 2.0 per cent of the total nitrogen. The absence of cystine in the most primitive plant belonging to the blue-green group is in striking contrast to its presence in the marine algæ, and may correlate with the botanical distinction between the two groups. Lysine is also absent in *Phormidium* as well as in the two most rudimentary of the marine plants examined.

Inasmuch as these analyses were made on the total proteins present in the algæ, the complete absence of any amino acid is biologically significant. It is interesting that *Sargassum* and *Chondrus* contain all the amino acids for which tests were made, except methionine.

The analytical methods employed in this study give quantitatively reliable values for cystine (11), tyrosine and tryptophane (12), histidine, arginine, and lysine (13), proline (14), and glycine (15). The methods developed by Brazier (16) for the separation of the monoaminomonocarboxylic acids yield only approximate results, and no emphasis is laid on the quantitative aspect of the values here reported for this class of amino acid. The same reservation applies to the separation of aspartic and glutamic acids. The alkali-labile sulfur method (11) for the determination of cystine was used in preference to the Sullivan or Folin-Marensi methods because of the presence in the hydrolysate of much colored material which would have interfered with the colorimetric determinations. The presence of carbohydrates rendered the Baernstein method for methionine determination inapplicable; the difference between the total organic sulfur and the labile



sulfur values has been accepted provisionally as a measure of the methionine content.

#### EXPERIMENTAL

*Phormidium* Specimen I was collected by Dr. J. B. Conant in northeastern Massachusetts during June, 1935. It contained a considerable proportion of diatoms. As the amount available was small, its analytical exploration was limited to a few amino acids. *Phormidium* Specimen II (*Phormidium valderianum*, Gom.), obtained through the kindness of Dr. W. Randolph Taylor, was collected by Dr. Earl T. Rose and Dr. Francis Drouet in July, 1936, from a pond near Falmouth, Massachusetts. It was free of diatoms. *Ulva* Specimen I was collected at Woods Hole by the Marine Biological Laboratory, during July and August, 1936. *Ulva* Specimen II (*Ulva lactuca*, L.) was collected in tidal water at Englewood, Florida, by Dr. H. H. Darby during July, 1937. It was rapidly washed with fresh water, immediately covered with alcohol, and protected from air in order to minimize chemical changes prior to extraction. *Laminaria* and *Chondrus crispus* were collected at Woods Hole during July and August, 1936. *Sargassum* (*fluitans* and *natans*) was collected at the Tortugas Islands by Dr. Darby during July, 1936, washed with fresh water, and immediately stored under alcohol. *Osmunda claytoniana*, collected in central Connecticut during July, 1937, was immediately crushed under alcohol.

Before analysis, the algæ were freed of lipids by extraction with cold alcohol, and dried to constant weight in air at room temperature.

The amounts of the various algæ used were: *Phormidium* Specimen I, 47 gm.; *Phormidium* Specimen II, 140 gm.; *Ulva* Specimen I, 1000 gm.; *Ulva* Specimen II, 100 gm.; *Laminaria*, 1000 gm.; *Sargassum*, 900 gm.; *Chondrus*, 525 gm.; and *Osmunda*, 100 gm.

The method for determining the amino acids was essentially the same for all plants. In early experiments, 25 per cent sulfuric acid was employed to hydrolyze the proteins; later, 20 per cent hydrochloric acid was found more convenient. Most of the amino acids were determined by isolation, cystine by the alkali-labile sulfur, and "methionine" by the difference between the organic sulfur and the alkali-labile sulfur. Tyrosine and tryptophane

were determined colorimetrically in separate samples of the tissues without extraction with formic acid.

A typical analysis follows. 900 gm. of dry *Sargassum* containing 12.4 gm. of total nitrogen were digested on the steam bath for 24 hours with 5 liters of 90 per cent formic acid. The cooled mixture was filtered through gauze and the insoluble residue was twice again treated in the same way with 3 liter lots of fresh formic acid. The total insoluble material when dried weighed 400 gm. and contained 3.44 gm. of nitrogen.

The combined formic acid extract was freed of traces of insoluble matter by centrifugation, and an aliquot containing 0.700 gm. of nitrogen was removed for the cystine determination. The remaining solution was concentrated *in vacuo* to remove most of the formic acid. The residue was taken up in 2 liters of 25 per cent sulfuric acid and heated on the steam bath for 3 days, when the ratio of amino nitrogen to total nitrogen was constant at 0.55. The cooled acid solution was diluted to twice its volume with water and filtered. The black "acid humin" precipitate weighed 270 gm. and contained 1.19 gm. of nitrogen.

The filtrate was concentrated and the last traces of formic acid were removed by the continual addition of water. The residue was diluted with water to 1 liter, and a nitrogen-free precipitate was discarded. Analysis of aliquots showed the presence of 6.75 gm. of total nitrogen and 0.600 gm. of ammonia nitrogen. The solution was then diluted to 4 liters and brought to pH 4.0 with lime. The calcium sulfate was filtered off and washed with hot water faintly acidified with sulfuric acid. The precipitate, which contained only 0.150 gm. of nitrogen, was discarded. The filtrate was rendered alkaline to phenolphthalein with calcium hydroxide; the brown precipitate was filtered off and washed with hot water. This "alkali humin" precipitate when dried contained 0.310 gm. of nitrogen. The alkaline filtrate, after removal of the free ammonia, was freed of calcium with oxalic acid and of sulfate with barium hydroxide, and concentrated to 1 liter; it contained 5.79 gm. of nitrogen. From this fraction there was removed 0.48 gm. of nitrogen for preliminary experiments, leaving 5.31 gm. of total nitrogen in the solution employed for the isolation procedures.

*Cystine*—The aliquot (0.700 gm. of nitrogen) removed from the original formic acid extract was evaporated to dryness and hydro-

lyzed by heating with 500 cc. of 20 per cent hydrochloric acid for 3 days on the steam bath. The acid humin was removed, and the filtrate concentrated to a syrup. This was diluted to 250 cc. and found to contain 0.616 gm. of nitrogen. Aliquots of 2 cc. were removed for the determination of total sulfur by the method of Zahnd and Clarke (17), preformed sulfate sulfur by precipitation as barium sulfate, and alkali-labile sulfur by the method of Blumenthal and Clarke (11). The total sulfur was 0.121 gm., the preformed sulfate sulfur was 0.074 gm., and the alkali-labile sulfur was 0.047 gm. The organic sulfur (the difference between the total sulfur and sulfate sulfur) corresponded closely to the alkali-labile sulfur, which is essentially ascribable to cystine, indicating the absence of methionine.

In the case of *Phormidium* there was no alkali-labile sulfur; the organic sulfur is referred to "methionine." Methionine could not be determined by the Baernstein method (18), since much volatile iodide was produced from contaminating substances. In the case of *Osmunda* the "methionine" represents the difference between the organic sulfur and the alkali-labile sulfur.

*Basic Amino Acids*—From the neutral hydrolysate, containing 5.31 gm. of nitrogen, there was removed an aliquot containing 0.212 gm. of nitrogen for the determination of the basic amino acids by the method of Block (13). The solution was concentrated to 25 cc., adjusted to pH 4 to 5 with dilute sulfuric acid, and diluted to 50 cc. The method was followed as given with the exceptions noted. For the precipitation of arginine monoflavinate, the pH was adjusted to 2 to 3. For the precipitation of lysine phosphotungstate the acidity was adjusted so as to correspond to 2 per cent sulfuric acid.<sup>1</sup>

The arginine monoflavinate weighed 0.207 gm.

*Analysis*— $(C_6H_{11}O_2N_4)(C_{10}H_8N_2SO_3)$ . Calculated. N 17.21  
Found. " 17.2, 17.2

The histidine diflavinate weighed 0.178 gm.

*Analysis*— $(C_6H_9O_2N_3)(C_{10}H_8N_2SO_3)_2$ . Calculated. N 12.52  
Found. " 12.5, 12.5

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<sup>1</sup> The solubility of lysine phosphotungstate in 2 per cent sulfuric acid is somewhat lower than in the 5 per cent acid recommended by Block.

The lysine picrate weighed 0.213 gm.

*Analysis*— $(C_6H_{11}O_2N_2)(C_6H_5O_2N_3)$ . Calculated. N 18.67  
Found. " 18.6, 18.6

In the analysis of *Ulva*, *Laminaria*, and *Phormidium*, no lysine was obtained in the above procedure. In these cases, attempts to precipitate it from the water-soluble fraction of the copper salts (16) were also unsuccessful. For example, the free amino acids of this fraction of *Ulva* hydrolysate, dissolved in 100 cc. of water, were treated with phosphotungstic acid. Precipitation, which would have taken place if more than 2.7 mg. of lysine nitrogen had been present (19), did not occur. Since this solution represented 16.1 gm. of the original formic acid-soluble nitrogen, less than 0.02 per cent of this can have existed in the form of lysine.

*Proline*—The major part of the neutral hydrolysate (5.04 gm. of nitrogen) was evaporated to 300 cc. and adjusted to pH 2 to 3 with sulfuric acid; arginine was removed as the monofluoroborate, and the filtrate was evaporated to 300 cc. The pH was adjusted to 1.0, and the solution was added to 21 gm. of ammonium rhodanilate  $[(Cr(CNS)_4(C_6H_5NH_2)_2)NH_4]$  in 130 cc. of methyl alcohol for the determination of proline, according to the method of Bergmann (14). After one recrystallization, analysis of the precipitate of proline rhodanilate yielded the correct amount of nitrogen. The weight of proline rhodanilate isolated, corrected for solubility, was 22.21 gm.

*Analysis*— $[Cr(CNS)_4(C_6H_5NH_2)_2](C_5H_9O_2N) \cdot H_2O$ . Calculated. N 16.2  
Found. " 16.3, 16.3

Pure proline was isolated from the complex salt by decomposition with pyridine.

*Analysis*— $C_5H_9O_2N$ . Calculated, N 12.17; found, N 12.1, 12.1

The filtrate from the proline rhodanilate was treated according to the method of Bergmann (14) with 20 gm. of Reinecke salt (20) and 1.3 cc. of pyridine. The product was decomposed with an excess of pyridine (14); the corrected yield of oxypoline was 1.750 gm.

*Analysis*— $C_5H_9O_2N_4$ . Calculated, N 10.69; found, N 10.6, 10.7

The filtrate from the oxyproline-reineckate complex was treated with excess pyridine, the pyridine rhodanilate and reineckate were removed, and the filtrate was made alkaline to phenolphthalein with calcium hydroxide. The filtered alkaline solution was concentrated to remove the pyridine and free ammonia; the excess calcium was removed with oxalic acid and the sulfate with barium hydroxide. The resulting neutral solution contained 3.77 gm. of nitrogen.

At this point isolation of the remaining amino acids was hindered by the presence of impurities of a carbohydrate nature. Advantage was taken of the method of Neuberg and Kerb (21) for the quantitative precipitation of the amino acids as the mercuric salts of their carbamates. Only proline and valine are reported to be incompletely precipitated from solution by this method. Proline had already been removed; the possibility of loss of valine was perforce accepted for the sake of determining the remaining amino acids.

The above solution containing 3.77 gm. of nitrogen was evaporated to 150 cc. and to it was added, in alternate portions and with vigorous stirring, 120 cc. of saturated sodium carbonate solution and 850 cc. of 25 per cent mercuric acetate solution, the mixture being kept just alkaline to litmus. The resulting suspension was poured into enough absolute ethyl alcohol to make an 80 per cent alcoholic solution. It was allowed to stand overnight, filtered, and washed with 80 per cent alcohol. The insoluble mercury precipitate contained 3.27 gm. of nitrogen (87 per cent recovery).<sup>2</sup> Both it and the soluble fraction were decomposed separately in warm dilute sulfuric acid solution with hydrogen sulfide. The soluble fraction contained 0.476 gm. of nitrogen, but as no amino acids could be isolated, it was discarded.

*Glycine*—The solution of the amino acids resulting from the insoluble mercury carbamates was freed of sulfate, and two aliquots, each containing 0.164 gm. of nitrogen, were taken for the determination of glycine as the trioxalatochromiate by the method of Bergmann and Niemann (15),<sup>3</sup> 3.0 gm. of potassium trioxalato-

<sup>2</sup> In subsequent determinations as much as 96 per cent of the nitrogen was recovered in this fraction by keeping the volume of the hydrolysate low.

<sup>3</sup> The details of this method were kindly communicated, prior to their publication, by Dr. Bergmann.

chromiate ( $[\text{Cr}(\text{C}_2\text{O}_4)_3]\text{K}_3 + 3\text{H}_2\text{O}$ ), as prepared by Lapraik (22), being taken for each portion. The amino nitrogen in the product, determined by the method of Kendrick and Hanke (23), amounted to 1.48 and 1.50 mg., the corrected average of which corresponds to 0.5 per cent of the total nitrogen.

*Dicarboxylic Acids*—From the remainder of the above solution, containing 2.94 gm. of nitrogen, the dicarboxylic acids were precipitated as their barium salts (containing 0.567 gm. of nitrogen) by the method of Jones and Moeller (24), and isolated as glutamic acid hydrochloride and aspartic acid (through the copper salt), after they were partially separated by means of their zinc salts (25, 26).

The total yield of glutamic acid hydrochloride was 1.966 gm.

*Analysis*— $\text{C}_5\text{H}_9\text{O}_4\text{N} \cdot \text{HCl}$ . Calculated, N 7.63; found, N 7.7, 7.6

The aspartic acid weighed 3.600 gm.

*Analysis*— $\text{C}_4\text{H}_7\text{O}_4\text{N}$ . Calculated, N 10.53; found, N 10.6, 10.5

*Leucine*—The barium salts of the amino acids soluble in alcohol were freed from alcohol by evaporation and decomposed quantitatively with sulfuric acid. They were converted to their copper salts by boiling with excess copper carbonate and fractionated according to the method of Brazier (16). The copper salts insoluble in water were decomposed and converted to their zinc salts. The zinc salt insoluble in cold water was decomposed and precipitated from a concentrated aqueous solution with alcohol. The resulting leucine weighed 0.150 gm.

*Analysis*— $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ . Calculated, N 10.69; found, N 10.6, 10.6

*Phenylalanine*—The water-soluble zinc salt from the above yielded 0.200 gm. of phenylalanine.

*Analysis*— $\text{C}_9\text{H}_{11}\text{O}_2\text{N}$ . Calculated, N 8.49; found, N 8.5, 8.5

*Alanine*—The copper salt fraction soluble in water and insoluble in dry methyl alcohol was decomposed with hydrogen sulfide and concentrated to a small volume. Some tyrosine which crystallized was removed, no attempt being made to estimate it at this point, as the tyrosine was determined colorimetrically, together with tryptophane (see below). The filtrate from the tyrosine crys-

tals was freed from histidine as the zinc-mercuric chloride complex, and lysine was precipitated as the phosphotungstate. The filtrate, freed of phosphotungstic acid and sulfuric acid, contained all its nitrogen (0.450 gm.) in the amino form, indicating the presence of only monoamino acids. Alanine and glycine were found; others may have been present, but only in small amounts.

The solution was made up to 250 cc. and was found to contain 0.403 gm. of nitrogen. Aliquots of 25 cc. each were removed and glycine precipitated as the nitranilate according to the procedure of Town (27). Each aliquot was evaporated to 10 cc. and made acid to Congo red; 30 cc. of absolute alcohol and alcoholic nitranilic acid were added till no more precipitate formed. The precipitated glycine nitranilate was centrifuged, the alcohol evaporated, and the excess nitranilic acid in the filtrate removed as the insoluble barium salt. The clear filtrate, containing 0.300 gm. of nitrogen, was concentrated to a small volume and treated with alcohol. The resulting precipitate, after one recrystallization from aqueous alcohol, gave the correct value for alanine.

*Analysis*— $C_3H_7O_2N$ . Calculated, N 15.73; found, N 15.7, 15.7

*Valine*—Valine was isolated by the method of Brazier (16) from the copper salts soluble in dry methyl alcohol. Some proline was isolated as its picrate (m.p.  $152^\circ$ ); this apparently represented the proline which had escaped precipitation as the complex rhodanilate, and for which allowance had already been made. The valine isolated weighed 3.386 gm.

*Analysis*— $C_6H_{11}O_2N$ . Calculated, N 11.96; found, N 11.9, 11.9

*Tyrosine and Tryptophane*—The method used was based on the Folin-Ciocalteu (28) colorimetric method as modified by Folin and Marenzi (12). A suspension of 50 gm. of dried *Sargassum* in 500 cc. of 10 per cent barium hydroxide solution, as recommended by Onslow (29), was heated on the steam bath for 4 days. Hydrolysis was then complete, as shown by a constant amino nitrogen to total nitrogen ratio of 0.55. The cooled mixture was filtered; the clear, nearly colorless filtrate and washings were freed from barium and concentrated to 100 cc., which contained 0.478 gm. of nitrogen. After decolorization by kaolin, 20 cc. aliquots were

found to contain 1.32 mg. of tyrosine nitrogen<sup>4</sup> and 1.39 mg. of tryptophane nitrogen.

The extract from 50 gm. of *Ulva*, treated as above, contained 0.749 gm. of nitrogen, but gave no color whatever in the tyrosine test. Since a distinct response can be obtained with 5 mg. of tyrosine under the same conditions, tyrosine cannot constitute more than 0.04 per cent of the formic acid-soluble nitrogen in *Ulva*.

#### SUMMARY

Amino acid analyses have been made on formic acid extracts of four marine algæ. In the lowest in the phylogenetic series of the algæ examined, *Ulva*, no methionine, tyrosine, or lysine could be detected. In the next, *Laminaria*, methionine and lysine are absent. The higher members, *Sargassum* and *Chondrus*, lack only methionine among the amino acids sought.

A higher autotrophic plant, the fern *Osmunda*, yielded all of the amino acids which were absent in the lower forms. *Phormidium*, a blue-green fresh water organism low in the evolutionary series but probably only distantly related to the marine algæ, was found to lack lysine and cystine.

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<sup>4</sup> An almost identical value (1.3 per cent) for tyrosine was obtained from the formic acid extract.



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# THE EFFECT OF ALLOXAN ON THE OXIDATION OF ALCOHOL BY VARIOUS TISSUES

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The effect of alloxan on tissue oxidation has not been studied, although this substance is an important constituent of the flavin molecule and hence of the yellow enzyme. Its reaction with tissues has been studied by Lieben and Edel (1) who showed that it combined with —SH groups in proteins. Cerecedo (2) isolated dialuric acid from the urine after feeding alloxan to a dog, and Labes and Freisburger (3) demonstrated convulsions after its injection into frogs. Recently Jacobs (4) showed that it caused a marked fall in blood sugar after intravenous injection into rabbits. Alloxan forms a reversible oxidation-reduction system with its reduction product dialuric acid and the potential of this system has been measured by Richardson and Cannan (5) who found an  $E'_0$  at pH 6.5 of 0.079 volt. Because of its relation to the flavins and because it can be reversibly oxidized and reduced, it was of interest to study the effect of alloxan on tissue oxidations.

## EXPERIMENTAL

The tissues were prepared by chopping them with scissors, grinding in a mortar with sand after the addition of 0.05 M phosphate buffer at pH 6.7, and then squeezing through muslin. The alloxan solution was made up in water, 1 mg. in 4 cc., and 0.1 or 0.2 cc. of this solution was added to the tissue in the Warburg vessel. The final volume of the liquid in the vessel was 2.0 cc., and thus the final concentration of alloxan varied from  $0.8 \times 10^{-4}$  to  $1.7 \times 10^{-4}$  M. Under these conditions the addition of alloxan caused a marked increase in the oxygen uptake by the livers of the rat, mouse, rabbit, and guinea pig, and a much smaller in-

crease in the oxygen uptake by the kidneys of these animals, and had no effect on the oxygen uptake by the brains. The effect on the guinea pig liver was the greatest and therefore this tissue was used. The livers of guinea pigs weighing approximately 400 gm. were prepared as described above, with 15 cc. of the buffer. 0.5 cc. of the resulting suspension was used in each Warburg vessel. The experiments were carried out at 37°.

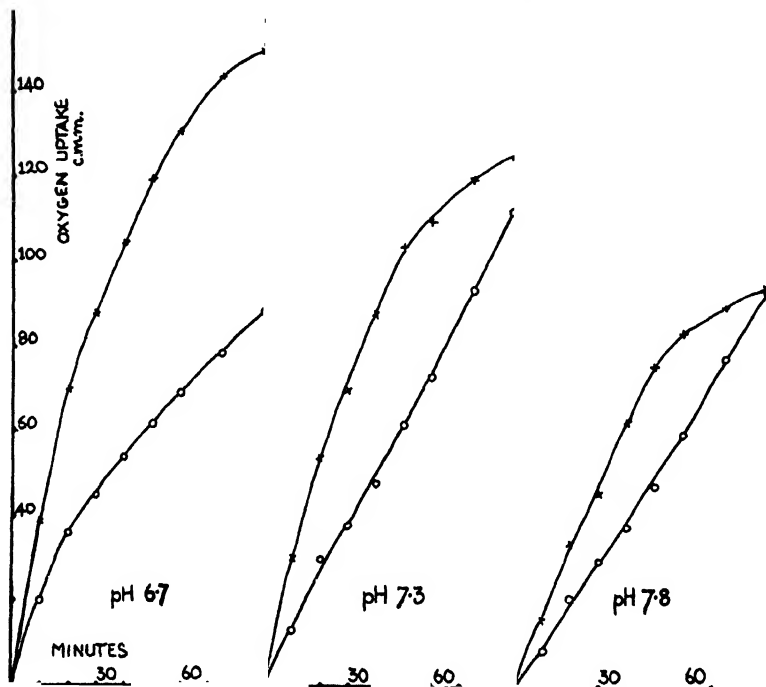


FIG. 1. The effect of  $1.7 \times 10^{-4}$  M alloxan on the oxygen uptake by guinea pig liver suspension at various hydrogen ion concentrations. The circles represent the liver alone, the crosses liver and alloxan.

*Effect of pH*—Fig. 1 shows a typical experiment. The acceleration of the oxygen uptake starts immediately and continues for 1 to 1½ hours. At the end of this time the slope changes and the rate of oxygen uptake is then slower than in the control. This effect occurs at the three hydrogen ion concentrations studied. Further addition of alloxan after the slowing has started has no

effect, which indicates that some substance in the liver has been used up. Because the percentage acceleration is greatest at pH 6.7, this pH was used for most of the following experiments. The  $\text{CO}_2$  production is only slightly increased, so that the R.Q. is lowered. In a typical experiment the normal R.Q. was 0.83 and with alloxan 0.69.

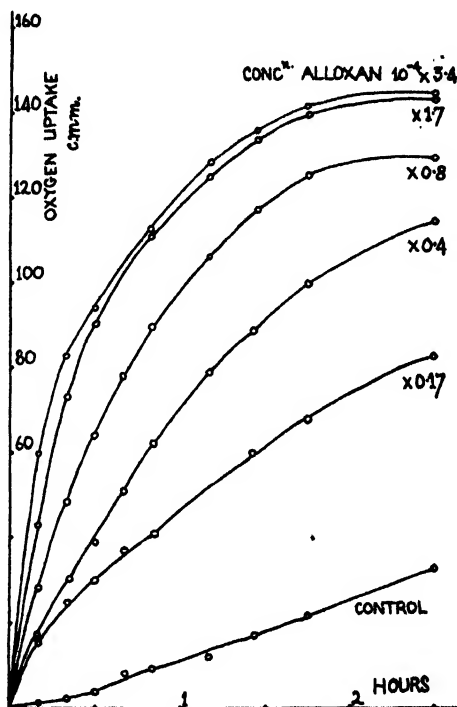


FIG. 2. The effect of various concentrations of alloxan on the oxidation of 1.0 mg. of ethyl alcohol by guinea pig liver at pH 6.7. The curves represent the oxygen uptake by the liver plus alloxan plus alcohol, from which is subtracted the oxygen uptake by the liver plus alloxan. The control represents the oxygen uptake by the liver plus alcohol, from which is subtracted the oxygen uptake by the liver alone.

*Effect of Addition of Substrates*—Various substances which are oxidized by the liver preparation were tested in order to see whether they affected the acceleration of oxygen consumption due to alloxan. These included lactate, citrate, pyruvate, suc-

ciate, amino acids, glucose, acetate, choline, tyramine, xanthine, and ethyl alcohol. None had any effect except alcohol. This substance, which is slowly oxidized by guinea pig liver to acetaldehyde, is oxidized 8 to 10 times more rapidly in the presence of alloxan. Fig. 2 shows the effect of different concentrations of alloxan on the oxidation of 1.0 mg. of alcohol. This experiment was performed at pH 6.7. At lower hydrogen ion concentrations similar curves can be obtained but the percentage acceleration is less, partly because the alcohol is oxidized by the liver somewhat more rapidly in more alkaline solutions. If less than 0.5 mg. of alcohol is used, the rate of oxidation is approximately doubled (see Table I).

TABLE I

*Effect of  $1.7 \times 10^{-4}$  M Alloxan on Oxidation of Different Concentrations of Alcohol by Guinea Pig Liver at pH 6.7*

Time	O <sub>2</sub> uptake					
	0.1 mg. alcohol	0.1 mg. alcohol + alloxan	0.2 mg. alcohol	0.2 mg. alcohol + alloxan	0.3 mg. alcohol	0.3 mg. alcohol + alloxan
min.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.
10	7	13	10	22	11	22
30	10	27	22	48	23	52
60	17	32	32	59	32	69
90	26	36	54	72	55	80
110	28	35	56	68	74	88

In order to determine whether the alcohol effect was really caused by an acceleration of the alcohol oxidation and did not involve the oxidation of any other substrate, it was necessary to study the alcohol concentration curve. For this purpose alcohol concentrations of 0.1 to 0.3 mg. were used. As shown in Table I, the extra oxygen uptake due to the alcohol in this concentration range is proportional to the amount of alcohol present. The actual uptake values show that between 1 and 2 atoms of oxygen are taken up per molecule of alcohol oxidized. The failure to get accurate uptake values is due to the difficulty of measuring alcohol accurately and also to the fact that some alcohol is lost in the vapor phase during the experiment. Similar loss also must affect the acetaldehyde formed, although most of the acetaldehyde is oxi-

dized further to the acid, probably by the xanthine oxidase. Table I shows, however, that in this concentration range alloxan accelerates the rate of the oxidation of the alcohol, but that the end-points are approximately the same whether alloxan is present or not. In other words the alcohol concentration is the limiting factor in this range. This indicates that alloxan accelerates the alcohol oxidation.

Further evidence that the alcohol is oxidized under these conditions is the fact that an aldehyde is formed. In a large scale experiment the aldehyde concentration was measured, after the oxidation had proceeded for a short while, by the method of Clift and Cook (6) and an increase in bisulfite-combining substance was shown in the vessel containing alcohol and alloxan. The increase was small, but this might be expected because the aldehyde when formed is rapidly oxidized further to acetic acid and could not be trapped by bisulfite or phenylhydrazine, as these compounds interfere with the alloxan effect. No attempt was made to isolate the acetic acid after the oxidation of the aldehyde because the occurrence of the oxidation is well known.

When greater amounts of alcohol are used, such as 0.5 to 1.0 mg., the extra oxygen uptake caused by the alcohol is no longer proportional to its concentration. This may mean that the time of the experiment is too short for the complete oxidation of the alcohol present. But this lack of proportionality also occurs when alloxan is present. It seems that after a certain length of time the alloxan effect disappears despite the fact that unoxidized alcohol is still present. Alloxan itself is not the limiting factor, for addition of more alloxan fails to restore the acceleration once it has disappeared. This evidence indicates that there is another factor in liver which is necessary for the alloxan effect.

*Effect of Age of Liver*—The following facts indicate that such a factor exists. In order to obtain the maximum effect with alloxan fresh tissue must be used. If the tissue is kept in the ice box for 3 hours, the acceleration due to alloxan is only about 70 per cent of the acceleration obtained on the fresh tissue. After 24 hours in the ice box the alloxan effect is negligible. Shaking the tissue at 37° for 2 hours before adding the alloxan also abolishes the effect. Dialysis in the ice box for 3 hours also causes the tissue to lose its ability to react with alloxan. These facts indicate that there

is a labile, dialyzable substance in the liver, the presence of which is necessary before alloxan can act catalytically on the oxidation of alcohol or increase the oxygen uptake of the tissue. Neither the age of the tissue nor dialysis affects the ability of the tissue to oxidize alcohol alone. Therefore this factor is not necessary for the normal oxidation of the alcohol by the tissue but only necessary for the alloxan action.

The oxidation of alcohol by tissues was first demonstrated by Battelli and Stern (7). Little was known about the mechanism of its oxidation for some time except that various authors showed that it was cyanide-sensitive (8, 9). Recently Lehmann (10) showed that cozymase was necessary for its oxidation by both yeast and tissues. Von Euler and Adler (11) confirmed this for yeast and showed that the yellow enzyme also acted catalytically on the oxidation. The fact that cozymase is necessary for the oxidation of alcohol by tissues shows that cozymase is still present when the alloxan effect has disappeared. The factor necessary for the alloxan action therefore cannot be cozymase; this is confirmed by the fact that addition of cozymase from yeast does not restore the alloxan effect in tissues which have been dialyzed or allowed to stand 24 hours.

*Specificity of Alloxan Effect*—Since alloxan belongs to a reversible oxidation-reduction system, it was possible that other similar systems such as methylene blue would act similarly in catalyzing the oxidation of alcohol. Addition of methylene blue in various concentrations had, however, no effect on the alcohol oxidation, although it increased the oxygen uptake of the liver alone, which shows that it has no toxic effect on the tissue. Various indophenol dyes acted like methylene blue in this respect. Pure lactoflavin used in various concentrations accelerated the oxidation of the alcohol 10 to 25 per cent under conditions in which alloxan caused an 800 to 1000 per cent acceleration. The catalytic action of alloxan can be considered therefore specific for this oxidation.

The effect of alloxan on the oxidation of other alcohols was tried. Methyl and propyl alcohols are oxidized very slowly by the liver preparation and alloxan has a small accelerating effect on their oxidation. The presence of butyl and amyl alcohols is without effect. Since ethyl alcohol is oxidized to acetaldehyde, the effect of alloxan on the oxidation of aldehyde was tried. Al-

though the conditions and concentrations were varied, no effect was observed. These experiments show that ethyl alcohol is the only substance which can be catalytically oxidized to a significant extent by alloxan in the presence of guinea pig liver.

*Effect of Inhibitors*—If alloxan is made up in phosphate buffer or is added to the buffer before the liver suspension, there was almost no catalytic action. The phosphate inactivates the alloxan. The liver, however, is suspended in phosphate buffer, and the alloxan is active in the presence of phosphate if it is added to it when tissue is present. This fact indicates that alloxan rapidly combines with some tissue constituent and is thus protected from the phosphate action. Pyrophosphate behaves like phosphate in this respect. Sulfate and chloride have no effect but bisulfite also completely inhibits the alloxan.

Cyanide, which inhibits the oxidation of the alcohol alone, also inhibits the accelerating effect of alloxan. It is difficult to decide whether this is due to the inhibition of a cyanide-sensitive system through which alloxan must act or whether it is due to a combination of cyanide with alloxan, thus inactivating it directly.

In the synthesis of flavin alloxan combines with dimethyldiaminobenzene, the two amino groups being in the *ortho* position in respect to one another. We have been unable to obtain this substance but have tried methyldiaminobenzene or 3,4-diaminotoluene. This when added to liver in concentrations of  $2 \times 10^{-4} M$  inhibits the alloxan effect about 70 per cent. The 2,4 isomer is only about half as effective as an inhibitor. This suggests that a combination with the amino groups on the ring prevents alloxan from combining with the tissue constituent. In this respect it is interesting that amino acids such as alanine, methionine, and cysteine when added to liver in comparatively large concentrations have no effect on the acceleration of oxidation due to alloxan despite the fact that under certain conditions alloxan will combine with amino and sulfhydryl groups.

#### DISCUSSION

For the normal oxidation of alcohol by the tissue dehydrogenase, cozymase is needed, as well as other transport systems which probably include cytochrome and indophenol oxidase, because of the cyanide sensitivity. In the guinea pig liver some factor



in this chain is for some reason limiting, so that the addition of alloxan can greatly accelerate the oxidation. This explanation is supported by the fact that the more dilute the liver, within limits, the greater the percentage acceleration by alloxan. If the normal transport systems were present in the liver in adequate amounts, the addition of alloxan would have little effect. The livers of other animals oxidize alcohol better than the guinea pig liver and the alloxan effect is correspondingly less and can only be demonstrated when the tissue suspension is dilute. The oxidation of alcohol by dried, washed yeast is unaffected by alloxan.

Keilin and Hartree (12) have shown that alcohol can be oxidized by hydrogen peroxide formed when some other substrate is being oxidized, the whole system representing a coupled oxidation. In the presence of alloxan the tissue produces some extra hydrogen peroxide, as judged by the extra methemoglobin formation when hemoglobin is added. This extra peroxide could be accounted for by the extra oxygen uptake of the tissue, for Bernheim and Michel (13) have shown that the methemoglobin formation under these conditions is a function of the oxygen uptake. Moreover, if hydrogen peroxide were responsible for the oxidation of the alcohol, hemoglobin would compete with the alcohol for the hydrogen peroxide and thus inhibit the alloxan effect. The presence of different amounts of hemoglobin has, however, no inhibiting effect on the acceleration by alloxan. The conclusion is that alloxan together with some dialyzable, labile substance in the liver can act with the alcohol dehydrogenase to catalyze the oxidation of ethyl alcohol.

#### SUMMARY

1. Addition of alloxan in concentrations of  $1 \times 10^{-4}$  M increases the rate of oxygen uptake of liver suspensions of various animals, the effect being most marked in the guinea pig liver. The effect is much less in the kidneys and is absent in the brain. The carbon dioxide production is only slightly increased.

2. Of all the substrates tried, only ethyl alcohol is oxidized more rapidly by the presence of alloxan. The acceleration varies from 800 to 1000 per cent.

3. The percentage acceleration is greater at pH 6.7 than at pH 7.8.

4. Lactoflavin, methylene blue, and certain indophenol dyes have no accelerating action on the oxidation of alcohol under these conditions.

5. The evidence indicates that the presence of a labile, dialyzable substance is necessary for the action of the alloxan.

6. Phosphate, if added to the alloxan before the alloxan is mixed with tissue suspension, inhibits the effect. Pyrophosphate also inhibits the effect, but chloride and sulfate are without action. Cyanide causes complete, and 3,4-diaminotoluene partial, inhibition.

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## THE PURIFICATION OF PROTHROMBIN\*

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In previous articles we have shown quantitative changes which occur in plasma prothrombin levels under a variety of conditions (1-3). Such quantitative studies require the use of stable, purified clotting reagents. The preparation of thromboplastin, fibrinogen, and prothrombin in a high degree of purity is even more essential when one wishes to study the fundamental mechanism of blood clotting. It is the purpose of this paper to describe the further purification of one of these reagents, prothrombin.

The ease with which prothrombin disintegrates under a variety of conditions has discouraged many workers in their efforts at purification. Also, when impure prothrombin is converted into thrombin, the latter tends to be inactivated by traces of antithrombic substances which may be present as contaminants. If prothrombin is adsorbed directly from whole plasma according to the technique of Fuchs (4), large amounts of antithrombin are also adsorbed. We find that this difficulty can be largely avoided if the prothrombin is first partially purified by the acetic acid precipitation technique of Mellanby (5). This prothrombin, which is now practically free of antithrombin, can be adsorbed on  $Mg(OH)_2$ . It is then eluted with  $CO_2$  and dialyzed against distilled water. The prothrombin remains in solution, while a number of the impurities present precipitate out. The solution thus obtained can be preserved for several days in the ice box without much disintegration of the prothrombin, and without spontaneous conversion into thrombin. When evaporated to

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dryness, it can be kept indefinitely. This product is considerably more active per unit weight of dry material than any previously described.

In converting the product into thrombin we use optimal quantities of  $\text{CaCl}_2$  and lung extract (1). Approximately 15 to 20 minutes are required for complete conversion. Once converted, the thrombin is quite stable. It can be kept for many days in the ice box without deterioration.

The quantity of prothrombin in the products obtained was determined by the method of Warner, Brinkhous, and Smith (1, 2). 1 unit of prothrombin is that amount which, when completely converted into thrombin, will clot 1 cc. of fibrinogen solution (2) in 15 seconds. By this method normal dog plasma can be diluted about 350 to 400 times before it is of unitary strength; it therefore contains 350 to 400 units per cc.

The following experiment, typical of many, illustrates our procedure. Ox blood was collected at the slaughter-house and mixed at once with 1.85 per cent potassium oxalate in the proportions of 1 volume of anticoagulant to 3 volumes of blood. After thorough mixing, the oxalated blood was placed in the ice box. On the following day the plasma was obtained by centrifugation. The latter, rather extensively diluted with oxalate solution, contained approximately 235 units of prothrombin per cc. 150 cc. were diluted with 1500 cc. of cold distilled water, and, by the addition of 1 per cent acetic acid (about 32 cc.), the pH was brought to 5.3, as determined with the quinhydrone electrode. As in Mellanby's procedure, the acidified, diluted plasma was then allowed to stand for 2 hours in the ice box while a sediment formed. The supernatant fluid was siphoned off and the precipitate was centrifuged sufficiently to pack it loosely. The precipitate was then shaken up in 75 cc. of a solution containing 0.075 per cent potassium oxalate and 0.86 per cent  $\text{NaCl}$ . The portion of the precipitate which did not dissolve was removed by centrifugation. The solution contained 36 per cent of the prothrombin originally present in the oxalated plasma. To adsorb the prothrombin, 10 cc. of  $\text{Mg}(\text{OH})_2$  suspension, prepared as previously described (2), were then mixed thoroughly with the solution, and thereafter separated with the centrifuge. The supernatant fluid, which still contained approximately 8 per cent of the prothrombin of the original oxalated plasma, was discarded. The  $\text{Mg}(\text{OH})_2$  was

then dispersed in 75 cc. of water by vigorous stirring, placed in a 500 cc. bottle, and shaken with  $\text{CO}_2$ . This was done at a pressure of 50 cm. of  $\text{H}_2\text{O}$ . In 10 minutes the solution became clear. After the elution with  $\text{CO}_2$ , the clear solution was placed in Visking casings (Visking Corporation, Chicago) and dialyzed for 15 hours at  $3^\circ$  against several changes of distilled water. The precipitate which formed during dialysis was removed with the centrifuge and discarded. Analyses of the supernatant fluid (77 cc. total) gave 1.3 mg. of solid material per cc. and 52 units of prothrombin per mg. of dry material. The yield at this point was estimated to be about 15 per cent.

Prothrombin solutions of this degree of purity are comparatively stable. During a period of 2 weeks they do not lose any of their activity when kept frozen at  $-35^\circ$ , and they retain about one-half of their activity when kept at  $2^\circ$ . Some prothrombin activity remains even if they are kept at room temperature ( $28^\circ$ ). If, however, such a solution is evaporated to dryness before a current of warm air, the residue retains practically all of its prothrombin activity when kept at room temperature.

Further purification can be accomplished by additional dialysis. This was illustrated with some of the above material, which was further dialyzed against repeated changes of cold distilled water, and finally against redistilled water over a period of about 8 hours. During this additional dialysis more precipitate formed; the latter was discarded. This dialysis procedure did not entail the loss of much prothrombin. The estimated final yield was 12 per cent. A small quantity of this fluid was evaporated on a watch-glass and found to contain 0.427 mg. of solids per cc. of fluid. Assay showed 151 units of prothrombin per mg. of solid material. In other words, 1 mg. of this material would clot 151 cc. of dilute fibrinogen in 15 seconds.

The dry material, described above, was practically colorless. It appeared to be homogeneous except for a few scattered threads of inactive material. The active material was readily soluble in water.

#### SUMMARY

A procedure is described for preparing prothrombin from ox plasma. The plasma is diluted, and the prothrombin precipitated by bringing the pH to 5.3 with acetic acid. The prothrombin is

then adsorbed on  $\text{Mg}(\text{OH})_2$  from a solution of this precipitate and eluted with  $\text{CO}_2$ . The eluate is then purified by prolonged dialysis against distilled water. A relatively stable and highly potent water-soluble material is obtained.

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# THE MOLECULAR STRUCTURE OF LIVER GLYCOGEN OF THE DOG\*

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Haworth and Percival (1) showed that the glycogen molecule consists of a chain of 12 anhydroglucose units joined by  $\alpha$ -glucosidic linkages between the 1st and 4th contiguous carbon atoms. There is therefore a close structural relation between this polysaccharide and starch, the essential difference being that the latter possesses a longer chain; namely, 24 to 30 glucose units (2-5). Recently Haworth and Isherwood (6) obtained from rabbits liver glycogen with a chain length of 18 glucose units. Bell (7, 8), applying Haworth's "end-group" assay method, examined glycogen from various sources and found it all to be of the same chain length, namely 12 glucose units, except in the case of glycogen formed in the rabbit liver after the ingestion of galactose, in which case it corresponded to a chain length of 18 glucose units. It has been shown that the glycogens with these two different chain lengths possess similar structures, exhibiting no essential differences in their general properties. In view of these variations in chain length, a study has been made here of liver glycogen obtained from another species; namely, the dog, an animal not hitherto investigated in this respect. The results obtained show that liver glycogen in this animal contains a chain length of 12 anhydroglucose units.

## EXPERIMENTAL

*Preparation of Glycogen*—The glycogen was obtained from dog liver by the following procedure. After removal from the animal,

\* Aided by a grant from the Research Board of the University of California, and by assistance from the Works Progress Administration.



the liver was rapidly cut into thin slices and put into boiling water. After the tissue had been boiled for about 15 minutes, the slices were finely ground and the extraction with boiling water repeated. The aqueous extracts were combined and the proteins precipitated by the addition of an equal volume of 10 per cent trichloroacetic acid. The glycogen in the filtrate was then precipitated in the presence of 40 per cent alcohol. This glycogen was redissolved and reprecipitated twice from the same concentration of alcohol. The glycogen was dried at 70° *in vacuo*, extracted with ether in an all-glass Soxhlet apparatus for a period of 12 hours, and then again dried at the same temperature to constant weight. Various lots of glycogen prepared in this manner contained between 0.01 and 0.001 per cent phosphorus. The properties of the dog glycogen were similar to those of glycogens obtained from rabbits and other sources. It was a white powder, which did not reduce Fehling's solution and gave a deep brown color with iodine. It was soluble in water, forming an opalescent solution. The specific rotation,  $[\alpha]_D$ , of an anhydrous sample was +194° (in water,  $c = 0.25$ ).

**Acetylation**—The glycogen was acetylated by Haworth, Hirst, and Webb's modification of Barnett's method (9). 2 gm. of well powdered glycogen were soaked for 30 minutes in 12 cc. of acetic acid through which chlorine gas had been previously bubbled for 1 minute. 10 cc. of acetic anhydride, through which sulfur dioxide was passed for 1 minute, were then added, and the mixture was maintained at 80°, with occasional shaking, for 1.5 hours. The clear solution was poured into a large excess of cold water, and the resulting precipitate washed with water, alcohol, and ether and then dried *in vacuo* at 40°. The yield of the triacetate was 85.3 per cent of the theoretical and had a P content of 0.004 per cent.

**Specific Rotation**— $[\alpha]_D = +170^\circ$  (in chloroform,  $c = 1$ )

**Analysis**— $(C_6H_7O_5(CH_2CO)_3)_n$ . Calculated.  $CH_2CO$  44.8

Found. " 45.0

The specific viscosity of the glycogen acetate, when 0.04 gm. was dissolved in 5 cc. of *m*-cresol, was 0.111. This corresponds to an apparent molecular weight of 4000, determined by Staudinger's formula with  $K_{sp} = 10^{-3}$  (10).

**Methylation**—The method of direct methylation, as described by Hassid and Dore (5) for methylation of canna starch, was employed. In this procedure preliminary acetylation is avoided

and the first stage of the process is carried out in a medium of carbon tetrachloride. The method was carried out thus: 15 gm. of finely ground glycogen were treated with a mixture of 125 cc. of carbon tetrachloride and 90 cc. of methyl sulfate and vigorously stirred with a mechanical stirrer for 15 minutes. 200 cc. of 30 per cent sodium hydroxide were then slowly added over a period of half an hour. 460 cc. of 30 per cent sodium hydroxide and 200 cc. of methyl sulfate were then simultaneously admitted into the reaction flask. The methylating reagents were added from two dropping funnels in portions of 3.3 cc. of methyl sulfate and 7.5 cc. of sodium hydroxide every 10 minutes. At the end of this process the carbon tetrachloride was evaporated, and the mixture cooled and almost neutralized with sulfuric acid, the reaction mixture being left, however, slightly basic. 400 cc. of water were then added, the mixture heated to 100°, and the partially methylated glycogen separated and dried. The methoxy content,  $\text{OCH}_3$ , of this product was 32 per cent. This partially methylated glycogen was then methylated in a medium of acetone by the method of Haworth and Percival (1). It separated as a solid and was remethylated. After nine such treatments it was dissolved in chloroform, evaporated to dryness, and extracted with boiling ether. The product thus obtained had a methoxy content of 44.8 per cent; further methylation did not raise this value. The specific rotation,  $[\alpha]_D$ , of the methylated glycogen was  $+208^\circ$  (in chloroform,  $c = 1$ ).

*Hydrolysis of Methylated Glycogen and Separation of Cleavage Products*—The methylated glycogen was hydrolyzed and the products of hydrolysis were separated according to the procedure of Bell (7). From 9.2 gm. of methylated glycogen, 0.805 gm. of 2,3,4,6-tetramethylglucose, 6.36 gm. of 2,3,6-trimethylglucose, and 1.1 gm. of dimethylglucose were obtained. These products were identified by their specific rotation and methoxy content.

	$[\alpha]_D$ degrees	$\text{OCH}_3$ found	$\text{OCH}_3$ calculated
2,3,4,6-Tetramethylglucose.....	+83	52.1	52.5
2,3,6-Trimethylglucose.....	+70	41.3	41.9
Dimethylglucose.....		31.2	29.8

\* In water,  $c = 1$ .

The yield of 0.805 gm. of tetramethylglucose from 9.2 gm. of methylated glycogen corresponds to an estimated chain length of about 12 glucose units.

#### DISCUSSION

It has been shown that the extent to which glycogen can be methylated closely approaches the theoretical value for the methoxy content of trimethyl glycogen; namely, 45.6 per cent (1, 7, 8). This agreement, however, is purely accidental, since it has been demonstrated here as well as elsewhere (6-8) that the cleavage products of methylated glycogen are a mixture of di-, tri-, and tetramethylglucose, and it is the average methoxy content of these three products that closely approximates the theoretical methoxy content of trimethyl glycogen.

The occurrence of dimethylglucose among the cleavage products of methylated glycogen is somewhat difficult to reconcile with the current views on the structure of glycogen (1). The uniform 1,4 linkage of a continuous chain of 12 or 18 glucose units should yield trimethyl- and tetramethylglucose as the products of hydrolysis of methylated glycogen. The fact that dimethylglucose is present in considerable quantities indicates that some hydroxyl groups are "blocked" and therefore not subject to methylation. As an explanation of the occurrence of the dimethylglucose, Haworth and Isherwood (6) offer the possibility of 1,6 "cross-linkages" between individual glycogen chains. The assumption of such "cross-linkages" would leave occasional glucose units with only two exposed hydroxyl groups, thus accounting for the dimethylglucose in the methylated glycogen. In this connection it is interesting to note the recent observations of Karrer and Escher (11) on the structure of cellulose, which is closely related to that of glycogen. These workers point to the fact that certain hydroxyl groups in the cellulose molecule cannot be methylated; they suggest that the "blocking" may be due to the formation of anhydrides by the elimination of 1 molecule of water for every 8 or 10 glucose units. If this occurred, it could hardly be detected by elementary analyses. Such a hypothesis might also explain the occurrence of dimethylglucose in the hydrolysis of methylated glycogen. This point, however, requires further investigation.

## SUMMARY

1. The molecular structure of glycogen from dog liver has been investigated by the "end-group" assay method. The methylated glycogen was hydrolyzed and quantitatively separated into 2,3,4,6-tetramethylglucose, 2,3,6-trimethylglucose, and dimethylglucose. The amount of tetramethylglucose obtained showed the glycogen from dog liver to be made up of chains of about 12 anhydroglucose units.

2. The significance of dimethylglucose among the products of hydrolysis of methylated glycogen is discussed.

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# THE ACTION OF ARGINASE ON NATURAL PROTEINS AND DERIVED PROTEINS OBTAINED THERE- FROM BY TRYPSIN AND PEPSIN

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Inasmuch as Edlsbacher and Bonem (1), Jansen (2), and Hunter and coworkers (3-5) have shown that arginase does not act on known arginyl compounds but does on arginine compounds in which the carboxyl group is free although the  $\alpha$ -amino group may be substituted and that arginase and urease actions may be used for the quantitative estimation of arginine, it follows that a quantitative study on the action of arginase on native proteins and derived proteins obtained therefrom by protease action may throw some light on the mode of linkage of arginine in these proteins and on the linkages attacked by the proteases. In this paper I present the results obtained by arginase action on gelatin, casein, egg white, and edestin, and on the peptic and tryptic products of hydrolysis of each.

That trypsin rapidly liberates arginine in the early stages of hydrolysis from gelatin, casein, and somewhat more slowly from edestin was shown by Hunter and Dauphinee (6) and that this change is produced more slowly by pepsin action on gelatin and casein was stated by Torbet and Bradley (7).

## EXPERIMENTAL

### *Materials and Methods*

**Proteins**—Gelatin (Ucopco gelatin, United Chemical and Organic Products Company, Chicago). 5.0 gm. of this finely powdered gelatin were dissolved in 60 cc. of distilled water in a 100 cc. volumetric flask; a drop of phenolphthalein was added and the pH adjusted to 8.5 by adding a few drops of 10 per cent NaOH. The solution was made up to 100 cc. volume.

Casein (Merck's after Hanmarsten). 4.0 gm. were suspended

in 50 cc. of distilled water in a 100 cc. volumetric flask; a drop of phenolphthalein was added, and 10 per cent NaOH added drop by drop until a pH of 8.5 was reached. The casein went into solution and the solution was then made up to volume.

Egg white (after McMeekin (8), 60 mesh). 0.20 gm. was weighed directly into 1 × 8 inch test-tubes or a gelatin capsule was adjusted to contain 0.20 gm. of egg white; the egg white could then be measured into each tube instead of being weighed.

Edestin. A commercial grade was used as well as some that was prepared by the author from defatted hemp-seed meal and recrystallized twice from dilute (1 per cent) sodium chloride solution by cooling to 4°.

*Enzyme Preparations*—Arginase was prepared from beef liver by the method of Hunter and Dauphinee (6). The three preparations used in this study were free from deaminizing action on the four proteins studied. These preparations also were free from urease activity.

Urease was prepared from Jack bean meal by the method of Koch (9). 1 cc. of a 1:5 dilution was used. The preparation was free from arginase activity.

Trypsin (U.S.P. pancreatin, Parke, Davis and Company). 0.4 per cent concentration was used.

Pepsin (Parke, Davis and Company, 1:10,000 activity). A 1 per cent concentration was used.

#### *Corrections for Blank Values and Methods of Analysis*

Arginase action was determined by the writer's modification of Hunter's (5) method. In all determinations arginase was allowed to act first and then followed by urease action, instead of allowing both enzymes to act simultaneously, as is sometimes practised by Hunter.

Corrections for blanks were obtained by the following control incubations and were applied in all arginase and urease actions on proteins. (1) Amide ammonia = ammonia obtained from the protein by omitting arginase and urease action. (2) Desamino or deaminase ammonia = ammonia obtained from the protein by omitting urease action and subtracting amide ammonia. (3) Free ammonia (arginase) = ammonia obtained from the arginase preparation by aerating arginase alone. (4) Free ammonia

(urease) = ammonia obtained from the urease preparation by the same procedure. (5) Urea ammonia in the arginase preparation = ammonia liberated from the arginase preparation by urease action and applying the corrections in (3) and (4). (6) Half free arginine nitrogen in the arginase and urease preparations =

TABLE I

*Per Cent of Total Arginine Found As Reactive Arginine before and after Protease Action*

Treatment	Trypsin series				Pepsin series			
Gelatin (4% solution, total arginine content 7.85 per cent of protein)								
	0 hr.	$\frac{1}{2}$ hr.	24 hrs.	10 days	0 hr.	24 hrs.	7 days	10 days
Protein found as reactive arginine without protease action.....	0.1	0	0	0.24	0.10	0.13		0.15
Same, as additional reactive arginine after protease action.....	2.96	3.92	5.6	5.56	0.62	0.99		1.04
Total arginine found as reactive arginine without protease action.....	1.3	0	0	3.0	1.3	1.6		1.8
Same, as additional reactive arginine after protease action.....	37.5	49.7	74.1		8.0	12.6		13.2
Casein (4% solution, total arginine content 3.74 per cent of protein)								
	0 hr.	$\frac{1}{2}$ hr.	24 hrs.	6 days	0 hr.	24 hrs.	7 days	10 days
Protein found as reactive arginine without protease action.....	0.9	0.93	1.18	1.30	0.53	0.60	0.60	0.60
Same, as additional reactive arginine after protease action.....	0.5	0.70	1.80	2.46	0.07	0.36	1.00	1.82
Total arginine found as reactive arginine without protease action.....	24.0	25.7	31.6	34.8	14.0	16.0	16.0	16.0
Same, as additional reactive arginine after protease action.....	13.4	18.9	50.0	65.8	2.0	9.6	27.0	50.0



TABLE I—*Concluded*

Treatment	Trypsin series				Pepsin series			
Egg white (4% suspension, total arginine content 4.92 per cent of protein)								
	0 hr.	24 hrs.	6 days	10 days	0 hr.	24 hrs.	7 days	10 days
Protein found as reactive arginine without protease action.....	0.3	0.29	0.45	0.45	0.29	0.30	0.32	0.31
Same, as additional reactive arginine after protease action.....	0.04	1.01	2.55	2.55	0.11	0.85	1.28	1.51
Total arginine found as reactive arginine without protease action.....	6.0	6.0	9.0	9.0	6.0	6.0	6.5	6.3
Same, as additional reactive arginine after protease action.....	0.8	20.5	51.8	51.8	2.2	17.2	26.0	30.7
Edestin (6.6% solution, total arginine content 14.17 per cent of protein)								
	0 hr.	24 hrs.	6 days	10 days	0 hr.	24 hrs.	6 days	10 days
Protein found as reactive arginine without protease action.....	0.19	0.37	0.41		0.26	0.26	0.26	
Same, as additional reactive arginine after protease action.....	2.73	6.08	9.67		1.66	1.88	3.09	
Total arginine found as reactive arginine without protease action.....	1.3	2.6	2.9		1.8	1.8	1.8	
Same, as additional reactive arginine after protease action.....	18.9	42.9	68.2		11.7	13.30	21.8	

ammonia liberated from the arginase preparation by consecutive action of arginase and urease preparations and by applying the corrections in (3), (4), and (5).

Of the values given above only that for amide nitrogen was appreciable and increased remarkably during proteolysis. Before proteolysis it ranged from 27 to 80 per cent of the total available

ammonia<sup>1</sup> for gelatin at pH 8.5, and from 76 to 119 per cent at pH 1.5; casein values ranged from 3.7 to 14.3 per cent at pH 8.5, and 7.4 to 18.1 per cent at pH 1.5; egg white from 8.2 to 12.7 per cent at pH 8.5, and 9.0 to 15.4 per cent at pH 1.5; edestin from 4.6 to 19.8 per cent at pH 8.5, and 5.9 to 37.9 per cent at pH 1.5. The maximum values obtained after proteolysis are 109 per cent for gelatin, 37.7 per cent for casein, 26.37 per cent for egg white, 50.3 per cent for edestin. These results are of the same general order as previously reported for casein (11).

TABLE II

*Summary of Absolute Weights of Arginine before and after Trypsin and Pepsin Action*

Proteins	Protein taken	Arginine found				
		In protein after complete acid hydrolysis	Maximum before trypsin action	Maximum after trypsin action	Made reactive by trypsin	Per cent total arginine found reactive after trypsin action
	mg.	mg.	mg.	mg.	mg.	
Gelatin.....	250	19.6	0.6	14.6	14.0	74
Casein.....	200	9.35	3.25	9.4	6.15	100.6
Egg white.....	200	9.8	0.9	6.0	5.1	61
Edestin.....	333	47.2	1.3	33.5	32.2	71
			Before pepsin	After pepsin	By pepsin	After pepsin action
Gelatin.....	250	19.6	0.35	2.58	2.23	13.0
Casein.....	200	9.35	1.49	4.68	3.19	50.0
Egg white.....	200	9.80	0.62	3.00	2.38	31.0
Edestin.....	333	47.2	0.84	10.28	9.44	22.0

The values for (3), (4), (5), and (6) were of the order of 0.12 and 0.14 mg. of nitrogen for 200 to 333 mg. of protein.

Each experiment was conducted in triplicate as follows: The protein was dissolved or suspended at pH 8.5 in NaOH for trypsin action and at pH 1.5 in HCl for pepsin action. In case the protein dissolved, aliquots were taken at different times; in case a sus-

<sup>1</sup> The values given by Mathews (10) are, for gelatin 0.4 per cent, casein 0.61 per cent, egg white 1.34 per cent, edestin 2.28 per cent.

pension was formed, the dry protein was weighed into tubes for the different incubation periods with and without proteases. At 0,  $\frac{1}{2}$ , 24 hours, and 6, 7, or 10 days after introduction of the protease solution the acidity of each series of tubes was adjusted to pH 6 and the tubes immediately placed in boiling water. After 5 minutes in boiling water the tubes were cooled and readjusted to pH 8.5 and the ammonia determined by (a) aeration as usual after action of arginase and urease, and (b) after arginase action without urease. Then (a) minus (b) represents ammonia obtained from arginine which was reactive to arginase, or one-half the reactive arginine nitrogen. From the data thus obtained the values in Tables I and II were calculated. The total arginine nitrogen for each protein was determined by arginase action on the protein after complete hydrolysis by hydrochloric acid.

#### DISCUSSION

If we assume that the ammonia liberated by the consecutive action of arginase and urease on any protein is a quantitative measure of a form of arginine with the free carboxyl group but not necessarily with the free  $\alpha$ -amino group, we arrive at the following conclusions.

1. Appreciable amounts of reactive arginine groups are present in casein and egg white at pH 8.5 and 1.5. At pH 8.5 casein reacts with 24 to 34.8 per cent of its total arginine, egg white with 6 to 9 per cent, gelatin with 1.3 to 3.0 per cent, and edestin with 1.3 to 2.9 per cent. At pH 1.5 the corresponding values are 14 to 16 per cent for casein, 6 to 6.3 per cent for egg white, 1.3 to 1.8 per cent for gelatin, and 1.8 per cent for edestin. The higher results obtained at pH 8.5 indicate that more hydrolysis takes place at that pH with the heated trypsin solution than at pH 1.5 with heated pepsin. Possibly the trypsin causes these changes in the short period taken to heat to boiling or the pepsin is destroyed more rapidly and completely than the trypsin.

2. Trypsin liberates additional reactive arginine more readily than pepsin. If we base the order of hydrolysis of the protein on the per cent of the total arginine made reactive by the trypsin, we have the sequence, gelatin, edestin, casein, and egg white in decreasing order. If we base the order on the mg. of arginine made reactive by trypsin, the order is, edestin, gelatin, casein,

and egg white (see Table II). Possibly the latter order is due to exposing a greater weight of combined arginine in edestin and less in gelatin, casein, and egg white.

3. Pepsin liberates reactive arginine groups most rapidly from casein and in decreasing order from egg white, edestin, and gelatin. The total reactive arginine found after pepsin action is 66 per cent for casein, 37 per cent for egg white, 23 per cent for edestin, and 14.5 per cent for gelatin. On the basis of the actual weight of the reactive arginine found after pepsin action, we have 11.17 mg. from edestin, 6.5 mg. from casein, 3.65 mg. from egg white, and 2.85 mg. from gelatin. This order is not the same as the total arginine involved for the respective proteins.

These results indicate that arginine is bound in more than one way in each of these proteins. It appears that some arginine linkings are much more resistant than others to protease action, but not necessarily in the same order toward trypsin and pepsin respectively. It also appears that these different types of arginine linkings occur in different amounts in the four proteins studied. A comparison of casein and edestin shows that of the 9.35 mg. of arginine in casein, only 1.5 mg. were reactive to arginase in the 0 hour digestion, and 9.4 mg. became reactive after 6 days incubation with trypsin; whereas in edestin, containing 47.2 mg. of arginine, 1.3 mg. were free to begin with and 33.5 mg. became so after 6 days incubation. The less complete hydrolysis of edestin cannot necessarily be due only to the larger amount of arginine available. It is just as likely and in fact more likely due to differences in the kinds of linkings involved. This is inferred from the different behavior of the four proteins toward trypsin. It is especially striking in the experiments on casein and egg white in which practically the same amount of total combined arginine, 9.35 and 9.8 mg. respectively, was used to start with and yet the percentages rendered reactive were 100 and 61 respectively. Equally important differences are shown by gelatin and edestin. Furthermore, the absolute and relative values for reactive arginine liberated were different for pepsin and trypsin. Finally, it must be acknowledged that the assumption that none of the arginyl groups reacts to arginase, but only free arginine or compounds containing arginine with free carboxyl groups, may not be true in all combinations of arginine.

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**Studies on Cystinuria. The Effect of Administration of Large Doses of Ascorbic Acid and of Methionine to the Cystinuric.**

BY JAMES C. ANDREWS, KATHLEEN CRANDALL ANDREWS, AND CHARLES B. RUTENBER. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia, and the Department of Biological Chemistry, School of Medicine, University of North Carolina, Chapel Hill*

Ascorbic acid (vitamin C) was administered in large doses (up to 6.0 gm. daily) to a cystinuric subject on a rigidly controlled diet. Because of the reducing potential of ascorbic acid it was thought possible that the cystine output might be modified by this procedure.

The resulting high content of ascorbic acid in the urine (up to 2380 mg. daily) made Sullivan determinations of cystine impossible. Precipitation of the cystine as the cuprous mercaptide gave values for cystine sulfur which showed no significant increase over the normal. Consideration of the optical activity values of these urine samples leads to the same conclusion.

The effect of large doses of methionine (up to 5.0 gm. daily) was also tested, since the same experiment performed on the same subject 3 years previously had failed to produce any marked increase in cystine output. In the present experiment the daily cystine output was increased to a maximum of 30 per cent. This result, obtained on a postadolescent subject, approaches more nearly that obtained by Brand and coworkers than was the case when the subject was preadolescent, but the increase in cystine output is still far less marked than that reported by them.

**The Source of Liver Fat.** BY H. M. BARRETT, CHARLES H. BEST, AND JESSIE H. RIDOUT. *From the School of Hygiene, University of Toronto, Toronto, Canada*

Deuterium-containing fats have been used to determine the source of the excess fat which appears in the livers of mice under a number of conditions. The methods used were similar in most details to those used by Schoenheimer and Rittenberg. A

gravimetric procedure for the determination of deuterium has been elaborated by one of us (H. M. B.).

It has been shown that the increase in liver fat which occurs in mice given anterior pituitary extract (Best and Campbell) by subcutaneous injection and in mice which have been fasted is due, in large part if not entirely, to transport of fat from the depots.

The fat accumulating in the livers of mice fed a high carbohydrate, choline-poor diet is apparently derived in large part from some source other than the fat depots. The carbohydrate of the diet is the most probable source of this fat.

Further evidence of the stability of the deuterium-containing fats has been obtained. There appears to be no more exchange between the deuterium atoms of the fat and the protein and glycogen molecules of the animal's tissues than would be expected to be produced by the small amount of deuterium oxide which appears in the body water.

**The Oxidation and Dismutation of Pyruvic Acid.** By E. S. GUZMAN BARRON AND CARL M. LYMAN. *From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago*

The metabolism of pyruvic acid by bacteria and animal tissues may proceed through either direct oxidation or dismutation, the predominance of either process depending mainly on the oxygen tension. In the presence of oxygen pyruvic acid is oxidized to acetic acid and  $\text{CO}_2$  by gonococcus (Barron\*) and by *Streptococcus haemolyticus*. In the absence of oxygen pyruvic acid is dismutated to lactic acid, acetic acid, and  $\text{CO}_2$  by gonococcus (Krebs†); it is dismutated to acetic acid and formic acid by *Streptococcus haemolyticus*. Contrary to Krebs'† contention, who postulated that pyruvic acid is oxidized in gonococcus by the anaerobic reaction and that the oxygen uptake after pyruvate addition is due to a secondary oxidation of the lactic acid, we have found these two processes to be independent of each other. Oxidation of pyruvic acid by gonococcus is inhibited considerably by NaF

\* Barron, E. S. G., *J. Biol. Chem.*, **113**, 695 (1936).

† Krebs, H. A., *Biochem. J.*, **31**, 661 (1937).

(0.01 M) and by  $\alpha$ -naphthol (0.001 M), while its dismutation is not affected at all. Under optimum conditions the rate of dismutation is greater than that of oxidation in these two species of bacteria. In pigeon brain (from Vitamin B<sub>1</sub>-deficient animals, and in the presence of synthetic aneurin) the rate of oxidation is greater than that of dismutation.

“

**Preparation of Prolactin Free from Other Pituitary Hormones and Preparation of a Mixture of Other Pituitary Hormones Free from Prolactin.** BY ROBERT W. BATES AND OSCAR RIDDLE.  
*From the Carnegie Institution of Washington, Station for Experimental Evolution, Cold Spring Harbor*

Prolactin when carefully prepared by the method of Bates and Riddle has no gonadotropic action, thyrotropic action, or adrenotropic action at dosage levels of 10 mg. daily in doves, 20 mg. daily in immature rats, or 50 mg. in the 3 month virgin rabbit.

We have found that prolactin is precipitated quantitatively from aqueous solutions with the hydroxides of zinc, copper, aluminum, and iron. Zinc and copper cannot be used to remove prolactin from dilute solutions when ammonium salts are present, e.g. urine. The residual prolactin in the supernatant liquid from a solution of 5 to 50 mg. of prolactin in 1 liter of H<sub>2</sub>O precipitated with 50 mg. of CuSO<sub>4</sub> or ZnSO<sub>4</sub> at pH 7 is less than 0.1 mg. as determined by the sensitive local crop test. The prolactin has been quantitatively recovered from the precipitate as determined by tryptophane concentration and physiological test.

When a copper salt (1 gm. per 10 gm. of protein) is added to the aqueous, isoelectrically soluble fraction from anterior pituitary extracts and adjusted to pH 7 with NaOH the small amount of prolactin present is quantitatively precipitated with some of the other physiologically active material. The active material in the supernatant solution at pH 7, after removal of the copper and other salts by dialysis around pH 3 to 5, is precipitated with ethanol or acetone. This copper-soluble precipitate gives a negative local crop test for prolactin with a dose of 1 mg. Since 0.2 microgram of our best prolactin preparations is the threshold dose (as we carry out the tests), it is thought that these copper-soluble fractions, which are very potent gonadotropic and thv-

rotropic fractions, contain less than 1 part of prolactin in 5000 parts of protein.

**The Iodine of Pituitary and Other Tissues.** BY EMIL J. BAUMANN AND NANNETTE METZGER. *From the Laboratory Division, Montefiore Hospital, New York*

Several investigators have published iodine analyses of pituitary glands which indicate that this organ contains a greater concentration of iodine than any other tissue of the body except thyroid. These observations have led to such interesting speculations as to how thyroid secretion influences the pituitary that we attempted to verify them. We could detect no iodine at all in single or pooled specimens of human pituitaries obtained from postmortem examinations where iodine medication had not been used in the 3 or 4 weeks before death. If, on the other hand, the patient had been treated with any form of iodine shortly before death, we usually recovered considerable iodine from the pituitary.

Practically no iodine was found in 5 gm. of beef pituitary or in 0.1 gm. of colloid from the pars intermedia—a part of the gland that has been supposed to be especially rich in iodine. The amount present is of the same order as that found in tissues other than thyroid.

**Basic Amino Acid Content of Posthemolytic Residue, or Stroma, of Erythrocytes.** BY ELIOT F. BEACH, BETTY NIMS ERICKSON, SAMUEL S. BERNSTEIN, AND HAROLD H. WILLIAMS. *From the Research Laboratory of the Children's Fund of Michigan, Detroit*

Posthemolytic residues, or stromata,\* of erythrocytes from various mammalian species have been examined for basic amino acid content by the method of Block.† Histidine was determined by application of the Kapeller-Adler colorimetric method of analysis to the purified histidine fractions. Arginine and lysine were estimated on the basis of the isolated flavianate and picrate derivatives, respectively.

\* Bernstein, S. S., Jones, R. L., Erickson, B. N., Williams, H. H., Avrin, I., and Macy, I. G., *J. Biol. Chem.*, **122**, 507 (1937-38).

† Block, R. J., *J. Biol. Chem.*, **106**, 457 (1934).

The lipid-free, vacuum-dried stromata contained from 12 to 14 per cent nitrogen and 0.8 to 0.9 per cent sulfur. Organic iron contents of the preparations indicated 5 to 10 per cent hemoglobin contamination.

The percentages of histidine in the stromata were: beef 0.6 to 0.8, horse 0.8, sheep 1.0, human 1.0 to 1.2. The arginine content varied from 4.9 to 5.4 per cent and the lysine from 3.5 to 3.8 per cent. The constancy of basic amino acid values in the various preparations suggests the presence of similar proteins in the stromata of the different species. The analyses indicate that the characteristic stromal protein is unlike other blood proteins, inasmuch as its basic amino acid composition differs from that reported for hemoglobin, fibrin, or serum protein.

**The Effect of Parenteral Injection of Purines, Methylated Purines, and Various Drugs upon Creatine-Creatinine Metabolism.**

BY HOWARD H. BEARD AND PHILIP PIZZOLATO. *From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans*

Adenine, xanthine, hypoxanthine, uric acid, and allantoin injected in small doses gave increases in muscle creatine in young rats from 43 to 58 per cent. Small doses of caffeine, theobromine, guanine, or prostigmine gave increases from 81 to 127 per cent. Thyroxine and epinephrine gave decreases in muscle creatine of 47 and 13 per cent, respectively. Small doses of curare, cocaine, morphine, ephedrine, atropine, pituitrin, veratrine, and acetylcholine were without influence upon the concentration of muscle creatine.

After injection of small doses of xanthine, theobromine, or caffeine, and deduction of the control output of creatine and creatinine for the 12 day period, the following amounts of "extra" creatine were excreted in the following 12 day experimental period: xanthine 32 mg., theobromine 49 mg., caffeine 53 to 71 mg. In only two out of twenty experiments was there a very significant increase in the excretion of creatinine. One of the caffeine studies showed a decrease of 100 per cent excretion of this substance.

**Conclusions**—(1) Creatine formation and excretion from the methylated purines is directly proportional to the number of

methyl groups present (up to three) in the injected substance. (2) The purines and prostigmin "stimulated" creatine formation and excretion. (3) Extra creatine was not transformed into creatinine in these studies. (4) The above results were not due to diuresis or excretion of muscle creatine into the urine. (5) The transformation of the imidazole group of the purines into creatinine is unlikely. (6) Use of the specific creatinine enzyme of Miller and Dubos\* showed that the color of the Jaffe reaction in our muscle filtrates and urines was due to creatinine alone.

### **A Quantitative Study of the Replaceability of Cystine by Various Sulfur-Containing Amino Acids in the Diet of the Albino Rat.**

BY MARY A. BENNETT. *From the Lankenau Hospital Research Institute, Philadelphia*

Last year the replaceability of cystine with some partially oxidized derivatives was reported. The compounds were fed in sulfur equivalents of 20 mg. of cystine daily to albino rats on a cystine-deficient diet (Dyer and du Vigneaud†). The results could not be interpreted quantitatively, since with this diet 20 mg. of cystine exceeded the minimum necessary for maximum growth.

In the first experiment reported here, cystine was fed in 20, 15, 12, 6, and 3 mg. portions daily to five groups of twelve rats each, to find the amount just adequate for maximum growth. 12 and 6 mg. of cystine were fed additional groups. Although 15 mg. of cystine daily were found adequate for maximum growth, the upper portion of the range proved less suited to quantitative measurements than the lower, in which slight differences in intake produced greater spread of the curves. Therefore 12, 6, 3, and 1.5 mg. of cystine were used in the succeeding experiment as the scale of reference.

In addition to the control and cystine groups, others were given cysteine, methionine, and cystine disulfoxide in amounts containing the sulfur equivalent of 12 and 3 mg. of cystine. All the curves representing 12 mg. equivalents fall in a group, as do those corresponding to 3 mg. The disulfoxide proved a possible excep-

\* Miller, B. F., and Dubos, R., *J. Biol. Chem.*, **121**, 457 (1937).

† Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **115**, 547 (1936).

tion, showing a slight lag as the experiment progressed. The results indicate that 1 molecule of cystine is metabolically equivalent to 2 of cysteine or methionine. The sulfur of cystine disulfide may be somewhat less readily available.

**Properties of Some Bacterial Peptidase Systems.** By J. BERGER AND MARVIN J. JOHNSON. *From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison*

A study of the peptidases of a number of common bacteria has shown striking differences between bacterial peptidases and previously known peptidases. Hydrolysis of some peptides by enzyme preparations from cells of *Leuconostoc*, *Propionibacterium*, and *Lactobacillus* species proceeds most rapidly at pH values from 5.5 to 6.0. Hydrolysis of peptides by some preparations is greatly accelerated in the presence of Zn<sup>++</sup>, Pb<sup>++</sup>, Cu<sup>++</sup>, and other divalent metal ions. However, some bacterial species (*Pseudomonas fluorescens* and *Proteus vulgaris*) show a specific magnesium activation of leucylglycine and leucyldiglycine hydrolysis similar to that of animal leucylpeptidase. *Leuconostoc mesenteroides*, and probably other bacteria, contain peptidases capable of hydrolyzing both optical forms of leucyl peptides. All previously studied peptidases hydrolyze only the naturally occurring *l* forms.

**The Isolation and Analysis of the Epicuticular Substance Cuticulin of the Silkworm, Bombyx mori.** By WERNER BERGMANN.

*From the Department of Chemistry, Yale University, New Haven*

The cuticle of insects consists of two primary layers, the thick, chitinous endocuticle and the very thin epicuticle. The biological importance of the latter lies in its impermeability and remarkable resistance to chemical action. The epicuticular substances have been named "cuticulins" by Wigglesworth. Such a cuticulin has now been isolated for the first time in a pure form. It was prepared from a large quantity of the last larval exuviae of the silkworm *Bombyx mori*. The cuticulin is a wax, closely resembling the cuticular substances of plants. Its principal components are paraffins of the probable order C<sub>25</sub>-C<sub>31</sub>, normal primary alcohols and fatty acids of the probable order C<sub>25</sub>-C<sub>31</sub>. Similar substances



have been isolated from the shells of eggs of *Bombyx mori* and from the silk fiber.

**Microdetermination of the Tyrosine Content of Protein Hydrolysates.** BY F. W. BERNHART. *From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis*

About 10 mg. of protein are hydrolyzed by heating in a boiling water bath with 0.2 cc. of 6 N sodium hydroxide for 4 to 5 hours. The hydrolysis is carried out in a 16 × 150 mm. Pyrex test-tube accurately calibrated at 10 cc. To the hydrolysate are added 0.3 cc. of 7 N sulfuric acid, 1.5 cc. of water, and 1.5 cc. of 15 per cent mercuric sulfate in 5 N sulfuric acid. The mixture is heated for 10 minutes in a boiling water bath, and cooled to room temperature. 1 cc. of 7 N sulfuric acid and 1 cc. of 0.2 per cent sodium nitrite are added with shaking. The volume is made up to 10 cc., and the contents are mixed. After centrifugation, the red color present is compared in the colorimeter with the color present in a standard tyrosine solution (2 cc. of a solution containing 0.2 mg. of tyrosine per cc. in 0.4 N sulfuric acid). A green filter (Wratten No. 61, Eastman Kodak Company) is used. Analytical results check closely with the values obtained by the method of Folin and Marenzi.

**A New Vitamin D in Cod Liver Oil.** BY CHARLES E. BILLS, O. N. MASSENGALE, K. C. D. HICKMAN, AND E. LEB. GRAY. *From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana, and the Research Laboratories, Eastman Kodak Company, Rochester, New York*

Previous studies showed (a) that the liver oils of different species of fish contain forms of vitamin D which react differently, per rat unit, on chickens, and (b) that the vitamins of fish oils can be separated, at different boiling ranges, by molecular distillation. The present study concerns the vitamins D of ordinary cod liver oil.

2 tons of cod liver oil were fractionally distilled, and the vitamin D fraction of lowest boiling point was redistilled. There was obtained a working quantity of the most volatile vitamin D, in a concentration about 10 times that of the original oil, as measured with rats.

The distillate and the original oil were quantitatively assayed

with rats and chickens. Per rat unit, the vitamin D of the distillate was markedly inferior to that of the oil for chickens. It produced almost no calcification when administered at levels where cod liver oil is effective. It was effective only at levels several times higher. This new vitamin D is the form which, on the basis of reasons elsewhere discussed, may differ from the chemically known forms in the absence of the usual side chain.

**A Photoelectric Photometer for Vitamin A Determination.** BY CHARLES E. BILLS AND J. C. WALLENMEYER. *From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana*

Light from an argon glow lamp is filtered through  $\text{NiCl}_2$  and red-purple corex. The filtered light, consisting solely of the two argon bands adjacent to the absorption maximum of vitamin A, passes through an absorption cell containing dilutions of vitamin A oils in isopropyl alcohol. Thence it goes through a variable slit to a cesium oxide, vacuum type, phototube. The phototube current, amplified, is indicated on a microammeter. A Pyrex filter, corresponding to a known unitage of vitamin A, is used to calibrate the instrument at each determination. The setting of the slit required to match this filter in microamperes is the measure of the amount of vitamin A in the absorption cell which replaces the filter.

The amplifying unit consists essentially of a constant voltage transformer, a power pack, a pair of Type 38 pentodes in a bridge circuit, two precision resistors, and a voltage-dividing resistor. The grid bias to the balancing tube can be varied so that the normal plate current of the amplifier is nullified. Thus any difference in plate current, indicated on the microammeter, represents a change in light at the phototube. The system is sufficiently stable to permit determinations at least as accurate as those made spectrographically. The complete unit is contained in a miniature radio cabinet.

**Effectiveness of Chondroitin As the Anti-Gizzard Erosion Factor Required by Chicks.** BY H. R. BIRD AND J. J. OLESON. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

Experiments previously reported have shown the effectiveness

of certain plant and animal products in preventing lesions of the gizzard lining in chicks fed various synthetic rations. Pork lung was found to be a particularly good source of the essential factor, but attempts at concentration met with little success, except that a potent preparation of a connective tissue protein, reticulin, was obtained. This finding led to experiments on other types of connective tissue. Doubtful results were obtained with samples of beef tendon and yellow elastic connective tissue but excellent protection followed the feeding of 10 per cent cartilage. The hexane extract of cartilage was ineffective. The feeding of chondroitin naturally suggested itself in view of the use of this compound in the treatment of stomach ulcer. It exerted a marked protective action against gizzard lesions when fed at levels of 3 and 5 per cent of the ration, and preparations of greater purity appeared to be more potent than crude chondroitin. Active chondroitin preparations have been obtained both by alkaline extraction and by 10 per cent  $\text{CaCl}_2$  extraction of cartilage. Experiments in progress are designed to determine the effectiveness of fragments of the chondroitin molecule and of related compounds. Glucosamine and glucuronic acid have been found ineffective at relatively low levels. Galactose fed as 3 per cent of the ration was not active.

#### **Determination of Iodine in Small Amounts of Thyroid Substance.**

By NATHAN F. BLAU. *From the Johnston Livingston Fund for Experimental Biochemistry, Department of Biochemistry, Cornell University Medical College, New York City*

A simple and rapid method for the determination of a few micrograms of iodine in thyroid material has been devised, requiring no elaborate equipment. Combustion of 1 to 25 mg. of the dry thyroid substance (or 5 to 100 mg. of fresh tissue) is carried out with KOH (0.5 to 1.0 cc. of saturated solution) and  $\text{KNO}_3$  (1 to 5 drops of 10 per cent solution) in a  $13 \times 100$  mm. rimless Pyrex test-tube. The contents are dried in an oven at  $110^\circ$ . The open end of the tube is then attached by an asbestos seal to the enlarged end of the intake tube of a 125 cc. Pyrex gas washing bottle containing a dilute solution of alkali. Gentle suction is applied at the outlet end of the absorber, while the lower portion of the combustion tube, containing the charge, is heated to cherry red-

ness in a Bunsen flame for 10 to 20 minutes. After the destruction of the organic matter, the combustion tube is broken into small fragments in the neck of a 125 cc. Claisen flask by a four-cornered tapering steel tool especially designed. The salts in the distilling flask are dissolved in the liquid from the gas washing bottle and an appropriate volume of water with which the absorber has been washed. The iodine is then distilled and the determination completed as described by Stimmel and McCullagh.\*

The accuracy of the method has been tested with KI, thyroxine, diiodotyrosine, and thyroid material of known iodine content. The values for iodine check closely ( $\pm 5.0$  per cent) with those obtained by the analysis of larger amounts of material by the Kendall iodine method. The procedure has been successfully applied to the analysis for total iodine and for the thyroxine iodine fraction of the thyroid glands of rats subjected to a variety of experimental conditions.

**Chemical Studies on the Neuroproteins. VI. Further Evidence for Sex Differences in the Amino Acid Composition of the Brain Proteins.** BY RICHARD J. BLOCK. *From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York*

A previous report (Paper III) has indicated that there is a sex difference in the amino acid composition of primate brain proteins. Since the publication of that report analyses for nitrogen, histidine, arginine, lysine, tyrosine, tryptophane, and cystine have been carried out on the proteins prepared from the brains of five more male primates, four more female primates, also four male, one castrate, and five female animals.

The results support the previous findings that male neuroproteins yield on the average somewhat more lysine than female brain proteins. Thus the average molecular ratio of arginine to lysine is 80:100 for twelve male primates against 86:100 for ten female primates. Likewise analyses of the proteins of pooled, whole animal brains give the following results: rats, arginine to lysine = ♂ 81:100, ♀ 86:100; rabbits, ♂ 79:100, ♀ 84:100; pigs, ♂ 82:100, ♀ 93:100; sheep, ♂ 84:100, ♀ 93:100. Dupli-

\* Stimmel, B. F., and McCullagh, D. R., *J. Biol. Chem.*, **116**, 21 (1936).

cate arginine and lysine analyses on cow and steer brain proteins gave 83:100 and 83:100 respectively.

The data also suggest that neuroproteins prepared from young mammals yield less histidine than those obtained from normal adults. Certain evidence appears to indicate that there may be a decrease in the histidine content of adult brain proteins associated with degenerative changes in the brain. Further work along this line is in progress. The procedure for the determination of the basic amino acids is described in detail.

**Experimental Liver Injury; Serum Phosphatase Activity in Relation to Other Findings.** BY AARON BODANSKY. *From the Chemical Laboratory, Hospital for Joint Diseases, New York*

As previously reported, a great increase of serum bilirubin and cholesterol, associated with an increase of serum phosphatase activity, follows the administration of single massive doses of *m*-toluylenediamine hydrochloride.\*

These changes are not associated invariably. By repeated administration of smaller doses of toluylenediamine hydrochloride, 20 to 40 mg. per kilo of body weight, the following changes were effected in adult dogs: Serum cholesterol rose as high as 3 times the initial level; serum phosphatase activity increased as much as 100 times; and the albumin to globulin ratio decreased from the initial level of about 2 to about 1.1. At the same time anemia was avoided, and serum bilirubin remained generally below 0.2 mg. per 100 cc.

**Studies on the Fractionation of a Factor of the Vitamin B Complex in Rice Polishings.** BY LELA E. BOOHER AND MARY LOJGIN. *From the Bureau of Home Economics, United States Department of Agriculture, Washington*

A factor present in rice polishings, distinct from thiamine chloride, riboflavin, and nicotinic acid and essential for growth and prevention (or cure) of erythroedemic dermatosis in rats, has been concentrated by use of group-precipitating reagents and adsorption on bentonite. This factor is apparently a basic substance but is not identical with any one or a combination of

\* Bodansky, A., *Enzymologia*, **3**, 259 (1937).

the basic substances, betaine, adenine, guanine, or choline, commonly known to be present in rice polishings.

**Autolysis of the Adrenal Glands.** BY H. C. BRADLEY AND S. BELFER. *From the Department of Physiological Chemistry, University of Wisconsin, Madison*

Study of the autolytic mechanism of adrenal tissue indicates that it does not diverge qualitatively or quantitatively from that of other gland structures such as liver or kidney. The exceptionally rapid postmortem disintegration of adrenal cortex structure therefore is not attributable to autolysis. It appears to be a cytolysis which occurs normally as the cortical cells mature, and represents the mechanism by which the cortical hormones are secreted into the blood stream.

**Growth Response to Sulfur Amino Acids.** BY ERWIN BRAND. *From the Departments of Biological Chemistry and Urology, College of Physicians and Surgeons, Columbia University, and the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York*

Rose *et al.* have shown that on diets containing amino acids as the sole source of nitrogen, young rats grow only if they receive sulfur in the form of methionine, cystine being ineffective. Since in cystinuria extra cystine is produced by cysteine as well as by methionine, but not by cystine, homocystine, or glutathione, the growth response of rats to these substances was investigated.

The various sulfur compounds were added to, or incorporated in, a basal diet which contained, with the exception of methionine, the amino acids established as essential by Rose plus glycine, arginine, and glutamic acid. Daily vitamin supplements consisted of cod liver oil, crystalline vitamin B<sub>1</sub>, lactoflavin, 75 mg. of milk concentrate, and 150 mg. of dried yeast (S content of yeast, 0.32 per cent). The average daily gain in weight was 2.1 gm. with *dl*-methionine (13 days), 1.1 gm. with *dl*-homocystine (14 days), 1.7 gm. with *dl*-homocystine (11 days) following a glutathione feeding period. Rats receiving cysteine (23 days), glutathione (14 days), cysteine + glutathione (13 days) failed to grow with or without the addition of ascorbic acid. These experiments

will be repeated with cysteine and glutathione supplied by injection.

The results are in agreement with the view that the conversion into cysteine is only one of the pathways of methionine metabolism, and that methionine has some other important function in the animal organism.

**Canine Cystinuria. IV.** BY ERWIN BRAND, GEORGE F. CAHILL, AND CHARLES A. SLANETZ. *From the Departments of Biological Chemistry, Urology, and Animal Care, College of Physicians and Surgeons, Columbia University, and from the Squier Urological Clinic of the Presbyterian Hospital, New York*

In continuation of the attempt\* to establish a cystinuric strain of Irish terriers, 120 dogs have now been raised and the urine of 101 of these dogs has been investigated. Two males (Nos. 4-19 and 4-20) were found to be cystinuric. These two dogs excrete about 150 mg. of cystine per day (20 to 30 per cent of the total S), whereas their normal litter mates (Nos. 4-21, 4-22, and 4-23) excrete 2 to 6 mg. of cystine per day (about 2 per cent of the total S).

It is hoped that progress in establishing a cystinuric strain will be more rapid in the future, since two homozygotic males are now available for back-crossing.

An exhibit is presented, consisting of charts, photographs, detailed breeding records, and cystine calculi from an Irish terrier and from a Scotch terrier.

**The Adsorption of Vitamin A by Sodium and Potassium Soaps When Formed in Situ in Highly Potent Oils.** BY H. N. BROCKLESBY AND C. C. KUCHEL. *From Pacific Fisheries Experimental Station, Fisheries Research Board of Canada, Prince Rupert, Canada*

A number of factors affecting the adsorption of vitamin A from potent oils by soaps formed *in situ* have been investigated.

Most important factors appear to be the water to soap ratios when alkalies just equivalent to free fatty acids present in the oil are added. With water to soap ratios for an oil containing 10

\* Brand, E., and Cahill, G. F., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **114**, p. xv (1936).

per cent free fatty acids of 100, 250, 500, 1000, 1500, the neutral oil lost 17.4, 30.4, 41.3, 50, and 54.3 per cent of vitamin A respectively, as determined by the antimony trichloride reaction. The above figures refer to sodium soaps formed at 95°. Potassium soaps caused much greater losses. Under similar conditions preformed soaps adsorb 6 per cent, while soaps formed *in situ* adsorb 39 per cent of the vitamin A.

Temperature of soap formation is important; at a water to soap ratio of 500 with sodium soaps losses of vitamin A in free oil at 20°, 55°, 80°, and 95° were 71.5, 63.1, 47.8, and 41.3 per cent respectively. Increasing free fatty acid content of oil causes increasing loss of vitamin A when oil is neutralized. Unsaturated free fatty acids on neutralization with either sodium or potassium hydroxide cause greater adsorption of vitamin A than the saturated homologues.

Further work on this subject is proceeding.

#### **Growth-Promoting Pituitary Extract in Thiamine Deficiency.**

BY J. C. BURKE AND A. R. MCINTYRE. *From the Department of Physiology and Pharmacology, University of Nebraska Medical College, Omaha*

Young male and female litter mate rats fed a diet free of thiamine, consisting of 58 per cent starch, 21 per cent casein, 14 per cent lard, 4 per cent salt mixture,\* 2 per cent cod liver oil, and 1 per cent autoclaved yeast concentrate, when injected with 0.5 cc. of growth-promoting pituitary extract (phyone) daily did not lose weight as early as their litter mates not receiving the injections. The animals deprived of thiamine began to lose weight on the 18th day; those receiving the injections did not lose weight until the 29th day. Phyone did not prolong the life of vitamin B<sub>1</sub>-deficient animals and their weight loss at death was the same as that of the controls. The animals receiving the phyone were all dead by the 42nd day; those deprived of vitamin B<sub>1</sub> survived a day or two longer; the series is too small to allow of definite conclusions as regards survival time. It seems unlikely that phyone can contain thiamine and we come to the tentative conclusion that phyone modifies the utilization of the stored thiamine.

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).



**Proof of Synthesis and Configurational Relationships of Abrine, the Naturally Occurring Amino-N-Methyltryptophane.** BY WILLIAM M. CAHILL AND RICHARD W. JACKSON. *From the Department of Biochemistry, Cornell University Medical College, New York City*

Amino-N-methyltryptophane was synthesized by Gordon and Jackson\* in 1935, and was demonstrated by them to promote the growth of rats ingesting a diet deficient in tryptophane. Shortly after the publication of this synthesis, Hoshino† reported that the crystalline substance, abrine, isolated from the seed of *Abrus precatorius* (jequirity seed), was an optically active amino-N-methyltryptophane. In order to compare abrine with the synthetic product, we completely racemized the optically active material prepared from jequirity seed, by treatment with a solution of barium hydroxide in an autoclave at an elevated temperature. Comparison of three different derivatives showed the racemized abrine and the synthetic amino-N-methyltryptophane to be identical. Using ketene gas as an acetylating agent, we succeeded in preparing monoacetyl derivatives of both abrine and racemized abrine. Acetyl-*dl*-abrina melts at 170–171°, and the acetylated natural abrine at 175–176°.

The optical configuration of abrine was also investigated. Abrine was methylated according to the Engeland procedure and the betaine obtained was found to be identical with the betaine prepared in the same manner from natural *l*-tryptophane. For comparison, hypaphorine, the naturally occurring and configurationally related betaine of tryptophane, was isolated from the seed of *Erythrina hypaphorus*. The betaines from all three sources possess a specific rotation of  $+113.5^\circ \pm 1^\circ$ . Inasmuch as tryptophane has been demonstrated to possess the configuration of other naturally occurring amino acids, we now have evidence that abrine like *l*-tryptophane and hypaphorine belongs to the *l* configurational series.

**The Metabolism of Mannitol, Polygalitol, and Styrcitol.** BY C. JELLEFF CARR, SYLVAN E. FORMAN, AND JOHN C. KRANTZ, JR. *From the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore*

\* Gordon, W. G., and Jackson, R. W., *J. Biol. Chem.*, **110**, 151 (1935).

† Hoshino, T., *Ann. Chem.*, **520**, 31 (1935).

The fate of the hexahydric alcohol, mannitol, and the two epimerides, 1,5-anhydromannitol and 1,5-anhydrosorbitol, was studied in the white rat and rabbit. Liver glycogen determinations, respiratory quotient measurements, and blood sugar determinations indicate that mannitol is capable of serving as a precursor of glycogen and the two anhydrides, polygalitol and styracitol, behave as physiologically inert substances.

**Vitamin E and Experimental Tumors.** BY CHRISTOPHER CARRUTHERS. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

Two pure strains of mice, Strains A and C-57, have been used in a study of the possible effects of vitamin E in the diet on the incidence of methylcholanthrene-induced tumors. The extent and frequency of spontaneous mammary carcinomas in the females of susceptible Strain A have also been examined.

The carcinogenic action of methylcholanthrene, dissolved in lard or spermaceti and injected subcutaneously into both strains of mice, was not significantly influenced when the diet contained little or no vitamin E or when this was administered in the form of a tested concentrate.

Conclusions regarding the effect of nutritional factors on the induction of tumors by potent cancer-producing hydrocarbons must be drawn with care; the vigorous action of these carcinogens may be independent of the nutritive state of the animal.

Methylcholanthrene induced more extensive ulceration in Strain A mice than in Strain C-57. In both strains the tumors were highly invasive. In Strain A mice methylcholanthrene markedly increased the incidence of non-metastatic "lung tumors."

Spontaneous mammary carcinomas arose in Strain A females whose vitamin E stores permitted only a "first litter" fertility. On a rancid vitamin E-free diet the incidence was very much lower. Whether this is due to the lack of vitamin E or to some other factor has not yet been determined.

Rapidly repeated pregnancies and pseudopregnancies favored the earlier incidence of mammary tumors. The occurrence of negative matings in Strain A females has led to an investigation of possible ovarian dysfunction.

**Amide Metabolism.** BY HERBERT E. CARTER, PHILIP HANDLER, FRANCIS BINKLEY, HAMILTON FISHBACK, WILLIAM RISSER, AND JAMES WEISIGER. *From the Laboratory of Biochemistry, University of Illinois, Urbana*

As the first step in an investigation of the fate of amides in the animal body, benzamide, phenylacetamide, and certain of their N-substituted derivatives were injected intraperitoneally into rabbits. The resulting toxic effects (kidney damage, death) together with the fact that no appreciable quantity of phenylacetic acid or its conjugation products was isolated from the urine of rabbits receiving the phenylacetamides indicate that hydrolysis of these amides in the rabbit occurs very slowly if at all.

Phenylpropionyl derivatives of *dl*-alanine, *dl*-phenylalanine, and *dl*-leucine were injected intraperitoneally into dogs. In each case an optically active phenylpropionyl amino acid and benzoic acid (free and conjugated) were isolated from the urine. Acetyl-, butyryl-, and valerylphenylalanine support growth in rats on an otherwise adequate phenylalanine-deficient diet. Trimethylacetyl- and  $\beta$ -methylvalerylphenylalanine are not utilized by the rat in place of phenylalanine.

The metabolism of amides in the animal body will be discussed in the light of these data.

**The Distribution of Body Water in the Partially Nephrectomized Rat.** BY ALFRED CHANUTIN. *From the Biochemical Laboratory, University of Virginia, University*

The volumes of extracellular and intracellular phases were determined in the muscles of normal and partially nephrectomized rats following (a) the intraperitoneal injection of isotonic glucose or hypertonic saline, (b) the ingestion of water or isotonic saline, and (c) the withdrawal of food and water. The procedure of Hastings and Eichelberger was used for these determinations.

After the intraperitoneal injection of glucose, the increase in the intracellular phase and the decrease in the extracellular phase were greater in the control than in the partially nephrectomized rats. On the other hand, there was a more marked decrease in the intracellular phase and increase in the extracellular phase in the partially nephrectomized rats following the remaining procedures outlined above.

These findings demonstrate that renal insufficiency *per se* may influence the water balance in the rat.

**Chemical Properties of the Lipid Fractions of *Bacterium tumefaciens*.** BY ERWIN CHARGAFF. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

In continuation of previous work on the chemical and biological properties of the various fractions of *Bacterium tumefaciens*, the etiological agent of plant tumors, which had been carried out in collaboration with Dr. Michael Levine of Montefiore Hospital, New York, the composition of the fat and phosphatide fractions of the microorganism was studied.

The fat (1.54 gm.), isolated by means of its solubility in acetone, had the following constants: iodine value 95.0, saponification value 201.2, acid value 53.2. On saponification it yielded 79.7 per cent of fatty acids and 7.1 per cent of unsaponifiable material. In the water-soluble fraction the presence of glycerol (2.5 per cent of the total fat) could be demonstrated. The unsaponifiable fraction consisted partly of one or more sterols which were precipitated as digitonides. These sterols may possibly be derived from the culture medium. The fatty acids, separated by fractionation of their methyl esters *in vacuo*, contained palmitic acid and oleic acid which was converted into stearic acid by catalytic hydrogenation. The major part of the fatty acids, however, consisted of a series of higher unsaturated acids, which in many respects were similar to the substances previously found in diphtheria bacilli.

The findings here discussed are of interest in connection with the conception that "structural specificity" of the chemical components of virulent bacteria is one of the prerequisites for their biological activity.

**Properties of the Lactic Acid-Racemizing Enzyme (Racemase) of *Clostridium butylicum*.** BY W. BLAKE CHRISTENSEN, W. H. PETERSON, AND MARVIN J. JOHNSON. *From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison*

Cells of *Clostridium butylicum* contain an enzyme capable of

converting either dextro- or levo-lactic acid into the racemic mixture. In old cultures, most of the enzyme is found in the medium, but in young cultures, the activity is largely confined to the cells. The enzyme is most active at pH 5. Since it has some activity even at pH 9, it apparently attacks the lactate ion rather than the free lactic acid. It has a high Michaelis constant (about 0.13). It is completely inhibited by 0.05 M cyanide. Since known lactic acid dehydrogenases have a low Michaelis constant and are not inhibited by cyanide, the enzyme does not seem to be a lactic acid dehydrogenase. Moreover, racemase preparations are free from lactic acid dehydrogenase activity. However, the possible identity of the enzyme with a dehydrogenase capable of attacking both enantiomorphs of lactic acid is by no means excluded. Such a dehydrogenase in the absence of an acceptor system would be expected to show racemizing activity.

Previous results from this laboratory indicating that the enzyme consisted of a thermostable and a thermolabile component have been found to be due to lack of accurate pH control in the early work. The nature of the enzyme, its distribution, and its possible relation to lactic acid metabolism, are being further investigated.

**Certain Reactions of Protein Films Prepared by the Langmuir Method.** BY G. H. A. CLOWES, W. W. DAVIS, AND M. E. KRAHL. *From the Lilly Research Laboratories, Indianapolis*

Protein films have been prepared and studied by suitable modifications of the methods of Langmuir and Blodgett, by use of a number of conditioning agents in conjunction with several types of barium-copper stearate films. Chief emphasis has been given to systems in which large molecules, *e.g.* salmine, with isoelectric points above pH 7, have been anchored to the barium stearate films and then allowed to adsorb other large molecules, *e.g.* insulin, with isoelectric points below pH 7. The effects of traces of metals and of hydrogen ion concentration have been particularly noted.

In addition, the effects of a series of substituted phenols on the formation of these films have been investigated, and an attempt has been made to correlate these effects with those exerted by the same reagents on cell respiration.

**The Non-Specific Nature of the Carbohydrate Portion of Horse Pseudoglobulin.** BY ROBERT D. COGHILL AND MARTHA CREIGHTON. *From the Department of Chemistry, Yale University, New Haven*

Ever since Rimington (1929, 1931) isolated 3.7 per cent of a polysaccharide from horse globulin, the question has remained open as to the rôle which the polysaccharide played in the immunological specificity of the protein. This problem has now been attacked in two ways.

Globulin has been exposed to the action of the pure recrystallized proteolytic enzymes pepsin, trypsin, and chymotrypsin. In each case the loss of protein nitrogen closely paralleled loss of specifically precipitable material, the loss being complete in the case of pepsin.

The polysaccharide in an impure but probably unaltered state was prepared without the use of barium hydroxide or acetates. This material, as well as the polysaccharide prepared by Rimington's original method, gives neither precipitin nor specific inhibition reactions with anti-horse globulin serum.

It is concluded from the evidence cited that the polysaccharide portion of horse globulin is not immunologically specific in the sense that the pneumococcus polysaccharides are, specificity of the protein being a property of the molecule as a whole.

On the basis of the acetyl content (1.2 per cent) and the polysaccharide figure (13.0 per cent) of the unaltered preparation, a new formula is suggested for the polysaccharide portion of horse globulin which is more in line with current views of the structure of chitin, mucins, and mucoids.

**Hepatic Ketogenesis and Ketolysis in Different Species.** BY PHILIP P. COHEN AND IRENE E. STARK. *From the Department of Physiological Chemistry, University of Wisconsin, Madison*

The ketogenesis of butyric acid and ketolysis of acetoacetic acid were studied by the use of liver slices from well fed and fasted rats, rabbits, and guinea pigs. Acetoacetic acid and  $\beta$ -hydroxybutyric acid were determined as a measure of ketogenesis and ketolysis, liver slices from the same animal being used for any given experiment.

In all three species studied to date liver slices from the well fed animals showed an appreciable destruction of acetoacetic acid other than by conversion to  $\beta$ -hydroxybutyric acid. Liver slices from animals fasted for 24 to 36 hours showed no significant ketolytic activity. The rate of reduction of acetoacetic acid to  $\beta$ -hydroxybutyric acid tends to be higher in the liver slices from well fed animals.

The rate of ketogenesis from butyric acid is not significantly greater with liver slices from fasted rats and guinea pigs than with those from well fed animals. Liver slices from well fed rabbits appear to have a lower rate of ketogenesis than those from fasted rabbits.

The spontaneous ketogenesis (no substrate added) is always significantly greater with liver slices of fasted animals.

**The Amphoteric Properties of Globin and Iodized Globin.** By EDWIN J. COHN, WILLIAM T. SALTER, AND RONALD M. FERRY. *From the Department of Physical Chemistry, Harvard Medical School, and the Collis P. Huntington Memorial Hospital of Harvard University, Boston*

Globin has been electrometrically titrated from pH 1.6 to 12.5. The acid-combining capacity is  $138 \times 10^{-5}$  mole per gm., or equal to the sum of the histidine, arginine, and lysine residues in the protein molecule. Approximately  $100 \times 10^{-5}$  mole per gm. dissociates acid to pH 5.0, and presumably represents carboxyl groups. As in the case of carboxyhemoglobin, dissociation at neutral reactions may be ascribed tentatively to histidine. The base combined between pH 7.5 and 12.5,  $89 \times 10^{-5}$  mole per gm., may be assigned to lysine and arginine or tyrosine.

The base combined at pH 12.5,  $102 \times 10^{-5}$  mole per gm., is  $11 \times 10^{-5}$  mole per gm. less than for carboxyhemoglobin. The acid-combining capacity is  $10 \times 10^{-5}$  mole per gm. less than reported for carboxyhemoglobin, and there are also differences in the strength of dissociation of acid and basic groups due presumably to the influence of the heme configuration.

It has been demonstrated for certain proteins, notably zein, that iodization of the equivalent of the tyrosine residues increases the strength of dissociation at alkaline reactions in the manner

to be expected from the much stronger acid properties of diiodotyrosine than of tyrosine. In the case of iodoglobulin no such effect was noted. When an amount of molecular iodine, equivalent to the tyrosine residues, was combined with globulin, the acid-combining capacity was unchanged, but the base combined at pH 12.5 was diminished by an amount approximately equivalent to the number of moles of iodine,  $I_2$ , combined with the globulin. Larger amounts of iodine increased the base and diminished the acid combined, but the total dissociation between pH 1.6 and 12.5 remained  $222 \times 10^{-6}$  mole per gm. as at lower iodine contents.

The nature of the influence of the heme configuration, and of iodine on protein dissociation is being further investigated.

#### **Further Evidence of Diabetic Tendencies in a Strain of Rats.**

BY VERSA V. COLE AND BEN K. HARNED. *From the Laboratory of Pharmacology, Woman's Medical College of Pennsylvania, Philadelphia*

Glucose tolerance tests on rats of the Wistar and Yale strains demonstrated a lower tolerance in animals of the latter strain. The data include tests on 300 male rats.

The technique consisted of a 15 hour fast, terminated by the intraperitoneal administration of 3.50 gm. of glucose per kilo, followed by blood sugar determinations at intervals of  $\frac{1}{2}$ , 1, 2, 3, and 5 hours. For comparable age groups after 90 days, the average blood sugar values of the Yale strain exceeded those of the Wistar strain by 30 mg. per cent at  $\frac{1}{2}$  hour, 55 mg. per cent at 1 hour, 60 mg. per cent at 2 hours, 70 mg. per cent at 3 hours, and 80 mg. per cent at 5 hours. The absorption of the glucose was identical in the two strains.

Analyses of individual blood sugar curves of the Wistar strain reveal only minor variations from the average. Similar analyses of the Yale strain show the presence of four curve types, one normal and three diabetic. Starting with rats 20 days old, we have analyzed and grouped our data in 10 day age increments. The results from our Yale strain show that diabetic curves rarely appear under 50 days of age but from this period the percentage of diabetic curves gradually increases to a constant value of approximately 70 per cent at 90 days of age.



**The Amino Acids Essential for the Adult Animal.** BY RALPH C. CORLEY, PAUL A. WOLF, AND ERNEST K. NIELSEN. *From the Laboratory of Biochemistry, Department of Chemistry, Purdue University, Lafayette*

Much is known about the nutritive significance of the amino acids for the growing animal; little about that for mature ones. A superior criterion of adequacy of the nitrogenous constituents of the diet is maintenance of nitrogen balance.

A mixture of the amino acids indispensable for growth, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine, in conjunction with a protein-free diet, has been found to keep white rats in nitrogen balance. Each of these amino acids is apparently indispensable in the adult organism, as the replacement in the diet of the nitrogen of any one of these amino acids by the nitrogen of arginine has resulted in a decided negative nitrogen balance. Restoration of the omitted amino acid to the diet, in each case, induced a return to nitrogen balance.

The basal diet containing 0.01 to 0.03 per cent nitrogen has been supplemented with a vitamin B concentrate (Harris). Urine by catheterization and feces by the aid of carmine markers have been separated into samples corresponding to 3 day periods. The mixtures of amino acids, in water, have been given by stomach tube. Animals have been in continuous experiment for over 4 months.

Studies are in progress to determine the minimal amount of each amino acid required for maintenance, the nutritive value of the foreign isomers, the interrelationships between the several amino acids, and the influence of the various regimens on the composition of the urine.

**The Quantitative Estimation of Vitamin A with the Photoelectric Colorimeter.** BY W. J. DANN AND KENNETH A. EVELYN. *From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina, and the McGill University Clinic, Royal Victoria Hospital, Montreal, Canada*

The Evelyn photoelectric colorimeter\* has been applied to the

\* Evelyn, K. A., *J. Biol. Chem.*, 115, 63 (1936).

quantitative measurement of vitamin A by the antimony trichloride reaction, and a method developed which combines speed and precision. From a series of readings taken with different volumes of the same solution of vitamin A it has been demonstrated that the standard deviation of the results is less than  $\pm 1.5$  per cent. This includes the errors inherent in the measurement of small volumes of chloroform solutions and in the instrumental readings.

Within the range of concentrations measurable in the colorimeter the relation of color depth to amount of vitamin A follows Beer's law exactly. Variation in the concentration of the antimony trichloride solution from 30 per cent (W/V) down to 15 per cent (W/V), or the presence of small quantities of water or alcohol in the solution, does not affect the initial depth of color produced.

The blue color begins to fade almost immediately after the reagent and the vitamin A solution are completely mixed; the rate of fading is least in solutions freshly prepared from dry antimony trichloride and dry, alcohol-free chloroform.

### **The Chemical Composition of Tissues in Adrenal Insufficiency.**

BY DANIEL C. DARROW AND HAROLD E. HARRISON. *From the Department of Pediatrics, Yale University School of Medicine, New Haven*

Certain tissues of adrenalectomized dogs and cats were analyzed for the principal electrolytes, nitrogen, and fat, so that the composition of extracellular and intracellular fluid could be calculated. The tissues of the cats showing symptoms of cortical insufficiency were compared with suitable controls; while specimens of muscle of the same dog obtained at biopsy were compared before adrenalectomy, during cortical insufficiency, and after cure with cortical extract and salt. The muscles of both cats and dogs during adrenal insufficiency show an increase in total water and a decrease in sodium, chloride, and nitrogen. The potassium in relation to nitrogen is increased from 10 to 20 per cent. In spite of the great decrease in concentration of sodium in serum the concentration of potassium in intracellular water remains normal, owing to increase in intracellular water. Total phosphorus shows no consistent change. In the cats, no dilution of cellular constituents was found in heart, liver, and kidneys and no increase in potassium

was demonstrated in these tissues. The kidneys show a decrease in sodium and chloride which is probably related to the defective reabsorption of these ions from glomerular filtrate. These studies indicate that a disturbance associated with retention of potassium in muscle is an essential feature of cortical insufficiency.

**The Effect upon Hematopoiesis of Variations in the Levels of Calcium, Phosphorus, and Iron in the Diet.** BY HARRY G. DAY, HAROLD J. STEIN, AND E. V. MCCOLLUM. *From the Biochemical Laboratory, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore*

Erythrocyte counts and hemoglobin determinations were made biweekly for 12 weeks on young rats weighing initially 100 to 120 gm., restricted to the mineral-deficient ration of Smith and associates\* and supplemented with minerals as follows: (1) basal ration plus vitamin supplements, (2) same as (1) plus 0.116 per cent  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , (3) same as (1) plus 2.086 per cent  $\text{CaCO}_3$ , (4) same as (1) plus 0.232 per cent  $\text{BeCO}_3 \cdot 4\text{H}_2\text{O}$ , (5) same as (1) plus 0.499 per cent  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , (6) same as (1) plus 3.537 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , (7) same as (1) plus 0.412 per cent  $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$ , (8) same as (1) plus 0.116 per cent  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and 0.232 per cent  $\text{BeCO}_3 \cdot 4\text{H}_2\text{O}$ . The sexes were equally divided and each animal received a total of 489 gm. of basal ration during the 12 weeks. The blood of Groups 3, 4, 5, and 8 was essentially normal, while that of Groups 1, 2, 6, and 7 was abnormal, being characterized by a polycythemia and concurrent anemia of mild degree. Since the basal ration and vitamin supplements used throughout contained only small amounts of calcium and relatively large amounts of phosphorus, it is concluded that (a) calcium does not have a unique function in hematopoiesis; and (b) excess dietary phosphorus interferes with iron utilization, probably owing to the formation of unabsorbable iron phosphate.

**The Riboflavin Requirement for Growth of the Rat.** BY PAUL L. DAY AND WILLIAM J. DARBY. *From the Department of Physiological Chemistry, School of Medicine, University of Arkansas, Little Rock*

\* Smith *et al.*, *J. Nutrition*, **12**, 373 (1936).

Albino rats which had received our flavin-deficient Diet 625\* for 2 weeks were given graded amounts of synthetic riboflavin. This was fed in weekly doses of 30, 60, 90, and 120 micrograms for 8 weeks or longer. Growth was roughly proportional to the amount of riboflavin fed between the 30, 60, and 90 microgram levels. The results of those experiments indicate that Diet 625 is satisfactory for the biological estimation of riboflavin when growth does not exceed 100 gm. in the 8 week experimental period. However, when growth exceeds 120 gm. in 8 weeks, some other essential substance may be the limiting factor. With our strain of animals, stock diet, flavin-deficient diet, and experimental technique 1 Bourquin-Sherman unit of vitamin G is equivalent to between 2 and 3 micrograms of riboflavin.

Rats given the flavin-deficient diet supplemented with 90 or 120 micrograms of riboflavin per week reached adult size and have remained in apparent health for more than a year, although growth proceeded at a slightly subnormal rate. Lower levels of flavin feeding failed to sustain growth to maturity.

**Ketolysis Versus Antiketogenesis As an Explanation for the Action of Carbohydrate on Ketonuria.** BY HARRY J. DEUEL, JR., LOIS F. HALLMAN, AND SHEILA MURRAY. *From the Department of Biochemistry, University of Southern California School of Medicine, Los Angeles*

When glucose was administered in doses of 1 mg. per sq. cm. of body surface to fasting rats in which an exogenous ketonuria was produced by the administration of butyric acid, the level of ketone body excretion was considerably decreased. Likewise, when this sugar was fed to fasting rats having an endogenous ketonuria produced by a previous high fat diet, the excretion of acetone bodies was largely abolished. The administration of ethyl alcohol in isodynamic amounts had no effect on the ketosis, although this alcohol is known to spare fat oxidation. A calculation of the amount of fat oxidized in fasting rats as compared with those receiving glucose showed that no appreciably different level in fat oxidation occurred, although the level of ketonuria varied greatly. Similarly it is shown that in a fasting human subject the

\* Day, P. L., and Langston, W. C., *J. Nutrition*, 7, 97 (1934).

ketonuria is practically abolished after the administration of 75 gm. of galactose, although the fat metabolism is decreased less than 10 per cent. On the basis of these results it is concluded that carbohydrate decreases ketonuria primarily by ketolysis rather than by the prevention of breakdown of fat.

**Metabolic Adjustments, Exercise, and Age.** BY D. B. DILL, H. T. EDWARDS,\* AND S. ROBINSON. *From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston*

Studies of males from 6 to 76 years of age indicate that the mobilization of carbohydrate is effective in maintaining the concentration of blood sugar near its basal value, regardless of age, in work that raises the level of oxygen consumption about 7-fold.

The proportion of carbohydrate utilized in such activity was low in boys and independent of age in men. Possibly the 15 hour fast that preceded the work depleted carbohydrate reserve to a greater extent in the boys than in the men, leaving the former with a less adequate supply of carbohydrate when exercise was undertaken.

5 minutes after brief, severe exercise blood sugar and lactic acid were moderately elevated in the boys and in the old men; the greatest increases occurred at intermediate ages. The ratio of increment in blood sugar to increment in lactic acid was high in young men, low in old men. For example, in thirty-six subjects with a mean age of 24 years, the blood sugar averaged 142 mg. per cent when the lactic acid was 60 mg. per cent. In thirty-seven subjects with a mean age of 48 years, the corresponding figures were 123 and 79.

**The Excretion of Androgenic Substances after the Administration of Testosterone.** BY RALPH I. DORFMAN. *From the Laboratory of Physiological Chemistry and the Adolescence Study Unit, Yale University School of Medicine, New Haven*

The excretion of androgenic substances in the urine and feces of adult male and female albino rats after the administration of testosterone has been studied. The hormone, dissolved in olive oil, was administered subcutaneously in one injection and the

\* Died, December 14, 1937.

urine and feces of these hormone-injected animals were collected for a period of 5 days. In control experiments, rats received olive oil injections and the excretions were collected in the same manner. The urine and feces were hydrolyzed with hydrochloric acid and extracted with benzene. The androgenic activity was assayed on the chick comb.

The control male and female rats excreted less than 0.3 international unit of androgenic substance during the 5 day test period. When 0.2 mg. of testosterone propionate was administered to either male or female rats, no androgenic activity was found in the excretions; however, when the quantity of testosterone propionate was increased to 10 mg., the equivalent of 1.0 to 1.5 international units of androgenic activity was obtained. The nature of this active material is being investigated.

**A Study of Hemochromogen Equilibria and a New Nomenclature for Hemoglobin Derivatives.** BY DAVID L. DRABKIN. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

The reaction of several oxidized\* or ferrihemochromogens† with  $\text{CN}^-$  has been studied spectrophotometrically. The spectra of the cyanide derivatives are readily distinguished from the spectra of the corresponding hemochromogens and are very similar to the typical spectrum of cyanmethemoglobin, both in the visible and ultraviolet regions. The spectra are not influenced appreciably by marked changes in pH, from 3 to 10. A mole of  $\text{CN}^-$  may unite per mole of  $\text{Fe}^{+++}$  in the hemochromogen solution. The addition of  $\text{Na}_2\text{S}_2\text{O}_4$  to the cyanide derivatives yields the corresponding ferrohemochromogens which can be converted to carbon monoxide hemochromogens,—reactions closely analogous with the formation of reduced hemoglobin from cyanmethemoglobin. The reaction of  $\text{CN}^-$  with ferric porphyrin to form the dicyanide hemochromogen must be distinguished from the above reactions of  $\text{CN}^-$  with various hemochromogens. Earlier spectroscopic studies of pyridine hemochromogen\* and dicyanide hemochromogen‡ have failed to make this distinction, and need clarification.

\* Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 89 (1935-36).

† Pauling, L., and Coryell, C. D., *Proc. Nat. Acad. Sc.*, **22**, 210 (1936).

‡ Barron, E. S. G., *J. Biol. Chem.*, **121**, 285 (1937).

A description of the structure and the spectra of these compounds call for more precise terms than "hemochromogen" for certain derivatives of the metalloporphyrins. The proposed nomenclature is based upon a slight modification of the accepted usage for coordinated compounds. Thus, for example, oxidized pyridine hemochromogen becomes dipyridino ferriporphyrin, and the reactions discussed above may be typified as follows: (1) dipyridino ferriporphyrin +  $\text{CN}^- \rightarrow \alpha$ -pyridino ferriporphyrin cyanide; (2)  $\alpha$ -pyridino ferriporphyrin cyanide +  $\text{Na}_2\text{S}_2\text{O}_4 \rightarrow$  dipyridino ferriporphyrin; (3) dipyridino ferriporphyrin +  $\text{CO} \rightarrow$  monopyridino monocarbonyl ferriporphyrin; (4) ferriporphyrin +  $\text{CN}^- \rightarrow$  dicyano ferriporphyrin (based on oxidation-reduction data<sup>†</sup>); (5) ferriporphyrin + excess  $\text{CN}^- \rightarrow \alpha$ -cyano ferriporphyrin cyanide (based upon analysis of the absorption spectra).

**Further Studies of Bromine Oxidation of Carbohydrates.** By F. L. DUFF, FAY SHEPPARD, AND MARK R. EVERETT. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

Optical rotations of 1 per cent sugar solutions, oxidized at 25°, have been investigated during 42 day periods. Equilibria of oxidized aldopentoses are shifted towards the  $\gamma$ -lactones in 48 hours. *Cis* arrangement increases the proportion of  $\gamma$ -lactone, oxidized *d*-ribose reaching the rotation of *d*-ribono- $\gamma$ -lactone.

Oxidized pentose antipodes give mirror optical rotation curves, but *d*-sorbose falls from +43° to +12° and *l*-sorbose from -43° to -57.5°. Reduction and acid production show that the *d*-antipode is most rapidly oxidized.

Glycerol and *i*-erythritol are oxidized largely to keturonic acids, while pentites, hexites, and heptites yield more ketose and non-reducing acid, ketose formation being the most rapid reaction. Oxidized *d*-mannitol and *d*-sorbitol solutions display almost identical properties. Optical rotation and fermentation indicate that at least 80 per cent of the reducing material from *d*-mannitol and the major portion from *d*-sorbitol are *d*-fructose.

$\alpha$ -Methyl-*l*-rhamnoside and  $\beta$ -methyl-*l*-arabinoside ( $[\alpha]_D^{20} = +245^\circ$ ) act like  $\alpha$ -glycosides. They are slowly oxidized without hydrolysis and form very levorotatory substances which lead to greater changes in rotation than oxidation of  $\alpha$ -hexosides. Hence,

oxidation for 7 days is better than 42 days for differentiating  $\alpha$ - and  $\beta$ -pentosides by rotational changes. These results and the hydrolysis of  $\alpha$ -methyl-*L*-arabinoside by emulsin indicate that  $\alpha$  and  $\beta$  forms of arabinosides have been misnamed.

Less than 10 per cent of the nitrogen is removed from *D*-glucosamine by bromine, indicating that the reducing product is an aminoketuronic acid.

**Raman Spectra of Choline, Betaine, Sarcosine, Glycylglycine, and Related Compounds.** BY JOHN T. EDSALL. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The influence of methylation of the amino group on the Raman spectrum of amino acids has been studied in glycine, sarcosine, and betaine. Certain powerful frequencies, found in the analogous amines and ammonium ions,\* appear in the amino acids with very similar values; in addition many new frequencies appear, owing to the greater complexity and asymmetry of the amino acid molecules. Unlike many other physical characteristics of molecules, the Raman spectrum is profoundly influenced by the introduction of methyl groups, and by their position. Even such closely related isomers as amino-*n*-butyric and aminoisobutyric acids give very different spectra, which thus serve to identify and differentiate them.

Choline chloride gives a beautifully clear spectrum with a large number of fine sharp lines, and a few powerful frequencies; it closely resembles the spectra of betaine and its hydrochloride.

Amino acids, like other carboxylic acids,† give characteristic frequencies which indicate the state of ionization of the carboxyl group, a strong line near  $1740\text{ cm.}^{-1}$  in the un-ionized group, and a very powerful line near  $1400$  in the ionized group. Thus the ionic state of such groups in a molecule can readily be determined by direct spectroscopic measurements.

Glycylglycine shows also a line at  $1700\text{ cm.}^{-1}$ , not found in any amino acid, and unaffected by ionization; this arises from the CONH group; other features in the spectrum of this peptide suggest variable molecular configurations due to the attraction of the charged groups.

\* Edsall, J. T., *J. Chem. Phys.*, **5**, 225 (1937).

† Edsall, J. T., *J. Chem. Phys.*, **5**, 508 (1937).



**The Lipid Distribution of Human Blood Platelets in Health and Disease.** BY BETTY NIMS ERICKSON, PEARL LEE, AND HAROLD H. WILLIAMS. *From the Research Laboratory of the Children's Fund of Michigan, Detroit*

Platelet samples have been prepared from blood of healthy men, women, and children, and from the blood of hemophiliacs. Post-absorptive blood samples were diluted 1:1 with chilled anticoagulant solutions (10 per cent sodium citrate; or, a solution containing 1 per cent sodium metaphosphate, 0.5 per cent sodium chloride, and 0.1 per cent dextrose). A suspension of intact platelets, virtually free of red and white blood cells, was achieved by repeated fractional centrifugation, the precaution being taken of keeping all equipment chilled. The platelets were separated by high speed centrifugation, washed twice with a 1:1 dilution of the anticoagulant solution, and dried *in vacuo* at low temperatures. A yield of 10 mg. of dried platelets was secured from each 50 cc. of blood; five or more individual preparations were pooled for lipid analyses by the microgasometric methods.

In man the lipid distribution of platelets indicates a composition similar to that of erythrocyte stroma. Expressed as per cent of dried platelets, the lipid distribution in thirty-four samples, apportioned into five groups for analysis, was: total lipid 15.4, phospholipid 11.4, free cholesterol 2.5, cholesterol esters 0.9, neutral fat 0.6, and protein 64.0. On the basis of choline determinations, 68 per cent of the phospholipids consists of cephalin.

Results of one group of five hemophiliacs indicate that the phospholipid fraction of their platelets is lower and the proportion of neutral fat higher. The percentage of phospholipid present as cephalin, however, appears to be normal.

**The Determination of Methemoglobin and Sulfhemoglobin with the Photoelectric Colorimeter.** BY KENNETH A. EVELYN AND HELGA TAIT MALLOY. *From the McGill University Clinic, Royal Victoria Hospital, Montreal, Canada*

From a study of the spectrophotometric absorption curves of methemoglobin, sulfhemoglobin, and cyanmethemoglobin, quantitative methods have been developed for their determination on the photoelectric colorimeter, by means of selective light filters. A brief summary of the theoretical foundations of the methods,

together with experimental evidence of their specificity and sensitivity, is presented.

**Chlorides in Biological Fluids.** BY WILLIAM G. EXTON AND ANTON R. ROSE. *From the Laboratory and Longevity Service of The Prudential Insurance Company of America, Newark*

The chloride ion is isolated as  $\text{AgCl}$  by a usual well known method. The  $\text{AgCl}$  is then dissolved in 10 per cent ammonia and reprecipitated by a reagent consisting of 100 mg. of powdered  $\text{K}_4\text{FeC}_6\text{N}_6 \cdot 3\text{H}_2\text{O}$  in 2 cc. of glacial acetic acid diluted to 100 cc. with 1 per cent ammonium sulfate. The silver ferrocyanide formed under these conditions has a homogeneous, stable, reproducible turbidity which we measure in a calibrated electroscopometer. Picric acid is used in deproteinizing. 0.20 cc. of blood is sufficient for a determination.

**Methemoglobin Formation by *Streptococcus viridans*.** BY ELLA H. FISHBERG AND HARRY BAUM. *From the Biochemical Laboratory of the Beth Israel Hospital, New York*

Many theories have been proposed to explain the methemoglobin-forming power of *Streptococcus viridans*, among them, the formation of hydrogen peroxide, enzyme action, etc. The demonstration by Conant of hemoglobin-methemoglobin as a definite oxidation-reduction system suggested some higher oxidation-reduction system within the *Streptococcus* itself. Because of the difficulties of working potentiometrically with hemoglobin we used indigo carmine as an indicator.

Indigo carmine was reduced with hydrogen in the presence of platinum; the hydrogen displaced by nitrogen from which the last traces of oxygen had been removed by passing over a copper spiral of low heat. Broth cultures of *Streptococcus viridans* introduced in a closed system at once produced a deep blue color, showing the presence of an oxidant within the cultures. This was repeated with plain broth under identical conditions and the leucoindigo carmine remained light. When this was repeated with various cultures of non-methemoglobin-forming *Streptococcus*, the leuco product remained in its reduced colorless state. Preheated cultures of *Streptococcus viridans* produced the same blue color, indicating a relatively stable oxidant. Ultrafiltrates

also gave a blue color. Extracts of washings from *Streptococcus viridans* cultures supplied by Eli Lilly and Company gave a similar blue color.

Titration of the culture with reduced indigo carmine gave within the physiological range an  $E'_0$  of +0.255 volt at pH 6.97 and an  $E'_0$  +0.210 volt at pH 7.69.

The ineffectiveness of sulfanilamide therapy in *Streptococcus viridans* may be due to the action of this higher oxidation-reduction system, since sulfanilamide is itself a potential oxidation-reduction system, while its effectiveness is striking in other *Streptococci* in which this system is absent.

**Studies on  $\beta$ -Glucuronidase.** BY W. H. FISHMAN. *From the Department of Biochemistry, University of Toronto, Toronto, Canada*

A method for the rapid preparation and purification of the enzyme, with beef spleen as the source, has been developed. This method involves the use of acetone and ammonium sulfate as precipitating agents. Fractional precipitation and extraction of the enzymes with suitable concentrations of ammonium sulfate solutions have yielded the best results.

This method differs in several respects from that reported by Masamune (1934) and Oshima (1936). The steps in this method involving autolysis of the spleen with saline, kaolin adsorption, and alcohol precipitation were omitted after thorough investigation.

The liberation of glucuronic acid from menthol and borneol glucuronides is taken as the basis for assaying the activity of  $\beta$ -glucuronidase. The increase in reducing power is determined by a modified Miller and Van Slyke method.

It has been shown that the  $\beta$ -glucuronidase content of various organs of the dog increases significantly after feeding large amounts of borneol, suggesting that  $\beta$ -glucuronidase is concerned in the *in vivo* synthesis of conjugated glucuronides.

**The Metabolism of Pyruvic Acid.** BY EUNICE FLOCK, JESSE L. BOLLMAN, AND FRANK C. MANN. *From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*

When 0.25 to 0.50 gm. of pyruvic acid, as the sodium salt, per

kilo of body weight per hour was given to dogs by continuous intravenous injection, the blood pyruvic acid showed an increase from the normal value of 1.5 mg. per 100 cc. up to from 6 to 16 mg. and returned to normal within 3 hours after the injection was discontinued. When the amount was increased to 1 gm. per kilo per hour, the blood level rose to as high as 70 mg. but again returned to normal unless a very deleterious alkalosis had intervened. The amount of pyruvic acid recovered in the urine varied from 5 to 23 per cent of the total. A striking increase in blood lactic acid accompanied the increase in pyruvic acid. In addition an appreciable though variable percentage of the injected pyruvic acid could be accounted for as lactic acid in the urine. No reduction of pyruvic acid to lactic occurred when sodium pyruvate was added to blood *in vitro*. The reverse reaction, the oxidation of lactic acid to pyruvic, did not occur when lactic acid was injected into the dog. Injections of glucose, levulose, and adrenalin produced small increases in blood pyruvic acid.

**Studies on the Prevention of Liver Cirrhosis by the Subcutaneous Injection of a Liver Preparation.** BY J. C. FORBES. *From the Department of Biochemistry, Medical College of Virginia, Richmond*

A liver preparation previously employed by us\* in the prevention of liver necrosis from carbon tetrachloride or chloroform administration has been used quite successfully in the prevention of liver cirrhosis from chronic carbon tetrachloride poisoning in rats.

When the material was given subcutaneously to rats in doses of 100 mg. per 100 gm. of body weight 18 to 24 hours before each poisoning, very good protection was obtained. The liver from none of the protected animals was severely damaged, while those from the controls were very cirrhotic. Doses of 50 mg. per 100 gm. of body weight, though exerting definite protective action, were not found sufficient to prevent moderate cirrhosis in some of the animals treated over a period of several months. In each of these experiments, however, the livers of the protected animals

\* Forbes, J. C., Neale, R. C., and Scherer, J. H., *J. Pharmacol. and Exp. Therap.*, **58**, 402 (1936). Forbes, J. C., and McConnell, J. S., *Proc. Soc. Exp. Biol. and Med.*, **36**, 359 (1937).

deviating the most from normal, exhibited less cirrhotic changes than the best of the controls.

**Chemo-Antigens and Carcinogenesis.** BY W. R. FRANKS AND H. J. CREECH. *From the Department of Medical Research, Banting Institute, University of Toronto, Toronto, Canada*

Serologically specific antigens are known to be formed by conjugation of chemical substances with proteins. A non-immunizing chemical component of a pathogen may be brought into the field of immunity by this means. This has been tested on cancer. 1,2,5,6-Dibenzanthracene was conjugated to protein. Dibenzanthracene isocyanate was formed from the meso amino derivative from which dibenzanthranlyl carbamido-proteins were formed. On injection into rabbits these proved antigenic, giving antisera showing cross-specificity for the prosthetic dibenzanthracene. The effect of this antigen on carcinogenesis was tested by immunizing mice. These and a control group were then injected with dibenzanthracene. In thirteen controls surviving over 180 days, nine developed invasive tumors and one leucemia. Similarly of twelve immunized mice four have developed tumors at the site of injection. Further, mice were injected with 1,2,5,6-dibenzanthraquinone followed by dibenzanthracene. Of four animals surviving over 180 days three have developed invasive tumors. There is some evidence that a degree of cross-immunity is given against carcinogenesis by methylcholanthrene.

The effect of such antigens on spontaneous carcinogenesis is under study. Indication that serological immunization by the above procedure should lead to protection against endogenous and exogenous carcinogens is compatible with present knowledge regarding immunity and carcinogens.

**The Chromoproteins of Photosynthetic Bacteria.** BY C. S. FRENCH. *From the Department of Biological Chemistry, Harvard Medical School, Boston, and the Research Laboratory of Physics, Harvard University, Cambridge*

Photosynthetic purple bacteria contain two groups of pigments—carotenoids, such as spirilloxanthin, and bacteriochlorophylls. Both pigments have been extracted with organic solvents and their chemical structure studied by several workers. By means of

supersonic vibration, it is possible to break open bacterial cells, liberating water-soluble proteins which carry with them the pigments, not in themselves soluble except in organic solvents. The infra-red absorption bands of the live bacterium *Streptococcus varians* at 795 and 855  $m\mu$  are found in exactly the same place and of nearly the same absolute height in the supersonic extracts, showing that the colored substance is not appreciably changed by the supersonic waves. The chromoprotein has an isoelectric point at about pH 3.7, as determined by infra-red photoelectric solubility measurements in buffer solutions. It can be precipitated by 0.5 saturated  $(NH_4)_2SO_4$  solutions. The extracts will not reduce  $CO_2$  with light and  $H_2$  but can act as a photocatalyst for ascorbic acid oxidation. Apparently the enzymes responsible for the Blackman reaction of photosynthesis are destroyed. Catalase activity is, however, present in the supersonic extracts. Adsorption of the chromoproteins on  $Ca_3(PO_4)_2$  in a Tswett column does not resolve the two pigments into bands of different color, indicating that both carotenoid and bacteriochlorophyll are attached to the same or similar proteins.

**Dispersion of the Dielectric Constant of Solutions of Urinary Proteins. I.** BY D. G. FRIEND, J. D. FERRY, AND J. L. ONCLEY. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

Measurements of the dielectric constant as a function of the frequency yield qualitative descriptions of dissolved fractions. In connection with studies of serum and tissue proteins, it seemed desirable to characterize also proteins excreted in the urine in certain pathological conditions.

Thus the urinary proteins from a case of nephritis have been investigated.\* The albumin and globulin fractions were first separated by precipitation with ammonium sulfate. The repeatedly reprecipitated globulin upon dialysis yielded a fraction insoluble at pH 5, closely resembling the  $P_1$  of Green. The electro-dialyzed pseudoglobulin (pH 6.5) had an extrapolated dielectric increment of 1.1 per gm. per liter. This value as well as the shape of the dispersion curve and the mean relaxation time ( $0.7 \times 10^{-6}$

\* We are indebted to Dr. J. P. O'Hare of the Peter Bent Brigham Renal Clinic for his courtesy and interest in supplying this material.

second) closely resembles the corresponding data for horse serum pseudoglobulin.

The fraction soluble in half saturated ammonium sulfate upon dialysis and electrodialysis had a pH of 5, a dielectric constant increment of 0.5 per gm. per liter, higher than any thus far observed for crystallized horse serum albumin, and a mean relaxation time in dilute solution of  $0.2 \times 10^{-6}$  second.

Characterizations of this kind are in progress for a series of proteinurias, and demonstrate the diverse nature of the proteins that are excreted.

**Effects of Thyroxine and Thyrotropic Hormone on the Weight and Nitrogen Metabolism of Dogs.** BY OLIVER HENRY GAEBLER AND PAUL BARTLETT. *From the Department of Laboratories, Henry Ford Hospital, Detroit*

In adult female dogs, single intravenous injections of 10 mg. of thyroxine produce changes in weight and nitrogen excretion which are reciprocally related to those observed in previous experiments with an anterior pituitary growth preparation under the same experimental conditions. Single injections of the growth preparation caused gains followed by losses; those of thyroxine caused losses followed by compensatory gains. There is, however, no evidence that the mechanism by which the initial effect of either hormone becomes reversed is the secretion of the other hormone.

Injections of thyrotropic hormone (antuitrin-T) in such experiments resulted in less decisive changes in nitrogen excretion and weight than those of thyroxine. The sulfur partition and purine excretion proved of no additional value as criteria. Creatine excretion, on the other hand, showed a marked response to injections of thyrotropic hormone. About 100 guinea pig units, given subcutaneously, caused a rise of 200 to 400 mg. in creatine excretion for several days, even though 1 cc. of Lugol's solution was added to the diet. The diets contained a constant amount of beef heart, and hence of creatine. Under these conditions the adult animals had a more constant creatinuria than is usually observed in young animals on creatine-free diets. The observed increase in the creatinuria is probably related to diminished storage of creatine, rather than to increased production of it.

**Effect of Parenteral Administration of Salt Solutions on Calcification in Vivo of Bones of Monkeys Made Rachitic by Light Deficiency.** BY HENRY J. GERSTENBERGER. *From the Babies and Childrens Hospital, Western Reserve University, Cleveland*

The monkeys were kept in an actinic ray-free environment and were fed a vitamin D-free diet, having in some instances a calcium to phosphorus ratio of 0.49 and in others one of 1.3; most of the animals received the latter. It has been found that on either of the two diets used a low phosphorus rickets develops, such as is customarily seen in full term human infants. It has also been established that the rickets so produced can be healed by exposure to ultraviolet light or by the administration of vitamin D in some form.

Twenty-two monkeys, made rachitic in the manner described above, have been used during the past year to determine whether parenteral administration of salt solutions would bring about calcification of the rachitic bones. It was found, as recorded by frequent roentgenographic studies and determinations of the calcium and phosphorus levels of the blood serum, that phosphate solutions of various kinds could do this; lactate and iodide salts were of no value. The studies included trials with salts of calcium, sodium, potassium, rubidium, and lithium.

**Isolation of Bilirubin from Hog Bile.** BY R. B. GIBSON AND R. C. LOWE. *From the Pathological Chemistry Laboratory, University Hospital, State University of Iowa, Iowa City*

Availability of hog bile suggests this material as a source of bilirubin, despite a low titer (Gibson and Goodrich) of 40 mg. per cent. Two simple procedures have given substantial yields.

1. 5 liters of chloroform are added to 20 to 25 liters of hog bile (best after standing 24 hours) and the bile-chloroform kept in the dark for 5 days with occasional agitation. The chloroform is drawn off and the extraction repeated. The extracts are dehydrated with sodium sulfate, filtered, and the bulk of the chloroform recovered by distillation and the remainder removed by a vacuum pan. The residue is extracted with ether, leaving crystals of bilirubin which are repeatedly washed with ether (50 cc. centrifuge tubes used) and recrystallized from chloroform in a continuous



extractor. The yield is about 2 gm. By-products are urobilin, cholesterol, and choleate.

2. If hog bile (strained or centrifuged) is saturated with chloroform, 5 cc. per liter, and kept in the dark for 4 days, crystals of bilirubin will separate. These are washed with water, alcohol, and ether and recrystallized from chloroform. The yield is about one-sixth the bilirubin content. The bile after preliminary crystallization may be processed by Method 1 and a further yield obtained.

### **Choline Esterase and Chemical Mediation of Nerve Impulses.**

By DAVID GLICK. *From the Laboratories of the Newark Beth Israel Hospital, Newark*

Superior cervical ganglia of the cat have been found to contain sufficient choline esterase to hydrolyze the acetylcholine liberated by a single nerve impulse in the theoretically least possible time of  $0.015 \sigma$ . However, the calculated theoretically longest possible time, based on the same data, is 8.3 seconds. The great divergence between these times indicates that a localization of the enzyme and substrate within the tissue should exist if the refractory period of  $2 \sigma$  is determined by the enzymic destruction of liberated acetylcholine. Calculations show that the extent of this localization would amount to the confinement of all of the enzyme and acetylcholine within  $1/420$  of the total volume of tissue. Attempts to determine the volume of the nerve endings in the ganglionic material failed, but it is not improbable that this volume is of the order of magnitude of  $1/420$  of the total volume. Possible objections to the theory that choline esterase determines the refractory period are discussed.

**The Synthesis of Aldobionides and the Relationship of the Molecular Rotations of Acetylated Derivatives of Glucose, Gentiobiose, and Cellobiose to Those of the Corresponding Uronic Acid Methyl Esters.** By WALTHER F. GOEBEL AND R. E. REEVES. *From the Hospital of The Rockefeller Institute for Medical Research, New York*

We have prepared acetohalogen derivatives of the methyl esters of 4- $\beta$ -glucuronosidoglucose (cellobiuronic acid), derived from the

hydrolytic product of the specific polysaccharide of pneumococcus Type III, and of 6- $\beta$ -glucuronosidogalactose (acaciabiuronic acid), the aldobionic acid of gum acacia. The methyl and *p*-nitrobenzylglycosides of hexaacetylcellobiuronic acid methyl ester are  $\beta$ -glycosides having a normal pyranose ring structure. Of four hexaacetates of acaciabiuronic acid methyl ester which have been prepared, two are believed to be pyranose and two furanose derivatives. Of two hexaacetyl methylglycosides of acaciabiuronic acid methyl ester, one is a furanoside, the other a pyranoside.

The molecular rotations of  $\alpha$ - and  $\beta$ -pentaacetylglucose, of  $\alpha$ -bromo- and chlorotetraacetylglucose, and of  $\beta$ -methylglucoside tetraacetate differ from those of the corresponding glucuronic acid methyl ester derivatives by a small and approximately constant amount. This striking relationship has also been found to exist between the values for the molecular rotations of certain acetylated derivatives of gentiobiose and cellobiose and their corresponding aldobionic acid methyl ester derivatives. Thus, among the aldoses at least, there is a definite relationship between the molecular rotation of the sugar and the corresponding uronic acid derivative. The conversion of the terminal acetylated primary alcohol group ( $\text{CH}_2\text{OAc}$ ) of acetylated aldoses to the corresponding carboxymethyl group ( $\text{COOCH}_3$ ) is accompanied by a slight change in molecular rotation of the uronic acid derivative. Further experimentation will determine whether this relationship holds true for the acetylated derivatives of sugars other than glucose, gentiobiose, and cellobiose and their corresponding uronic acids.

**The Availability of *d*- and *dl*-Amino-*N*-Methyltryptophane for Growth.** BY WILLIAM G. GORDON. *From the Department of Chemistry, Stanford University, California*

Previous experiments have shown that *dl*-amino-*N*-methyltryptophane is capable of stimulating growth in rats subsisting on a diet deficient in tryptophane. More recently Hoshino isolated *d*-abrine from jequirity beans and identified this substance as *d*-amino-*N*-methyltryptophane. Jackson and Cahill have now racemized the active material and have established the fact

that racemized abrine is identical with synthetic *dl*-amino-N-methyltryptophane (personal communication from Dr. R. W. Jackson).

The present report deals with a comparison of the efficacy of *d*- and *dl*-amino-N-methyltryptophane as substitutes for tryptophane. Young male rats maintained on a tryptophane-deficient diet were fed the basal ration supplemented with (a) 50 mg. per cent of *l*-tryptophane, (b) an equimolecular quantity of *d*-amino-N-methyltryptophane, and (c) an equimolecular quantity of *dl*-amino-N-methyltryptophane. The growth curves obtained demonstrate that *d*-amino-N-methyltryptophane is practically as efficient as *l*-tryptophane in stimulating growth. The *dl* derivative, on the other hand, is decidedly less efficient.

The results already at hand indicate that the naturally occurring *d*-amino-N-methyltryptophane is well utilized by the rat and may serve as an adequate substitute for tryptophane for purposes of growth; the *l* antipode apparently cannot be utilized in this manner.

Further feeding experiments with different quantities of the N-methylamino acid supplements are now being carried out.

**The Globulin Fractions of Antipneumococcus Serum.** BY ARDA ALDEN GREEN. *From the Department of Pediatrics, Harvard Medical School, Boston*

The globulins of antipneumococcus horse serum have been separated according to the methods previously employed by the author on normal horse serum. The relative concentrations have been compared and electrochemical studies carried out on the antibody-containing globulins.

**The Polarity of Aminocyclohexane Carboxylic Acids.** BY JESSE P. GREENSTEIN AND JEFFRIES WYMAN, JR. *From the Department of Physical Chemistry, Harvard Medical School, Boston, and the Biological Institute, Harvard University, Cambridge*

Relations between certain physicochemical properties and the distance between the polar groups of homologous aliphatic  $\omega$ -amino acids are likely to be obscured by the free rotation of C—C bonds in the chain. It was therefore considered desirable to investigate the properties of dipolar ions in which the possi-

bility of free rotation was reduced to a minimum. Two classes of substances fulfil this condition; namely, the benzbetaines, studied by Edsall and Wyman,\* and the cyclohexane amino acids.

The *o*- and *p*-aminobenzoic acids, owing to resonance, exist chiefly in the un-ionized state. On the other hand, the *m* derivative exists to a considerable extent in the dipolar condition. When all three forms are completely hydrogenated, they are converted almost entirely into the dipolar ion state as shown by their very high melting points, increased aqueous solubility, and ability to increase markedly the dielectric constant of their solutions. The latter property follows the usual conditions whereby  $D = D_0 + \delta C$ .

The hydrogenation of each aminobenzoic acid further gives rise to either *cis* or *trans* isomers, depending on the method of reduction. In HCl solution the *p* acid yields the *cis* form ( $\delta = 61$ ), whereas the *m* acid gives rise to the *trans* form ( $\delta = 62$ ). The calculated average geometric distance, based on x-ray data, between amino and carboxyl groups in these two forms is respectively 4.9 Å. and 5.1 Å. When the dipole distance,  $R$ , is calculated from the equation  $R = \sqrt{\delta/2.3}$ , there is obtained for the former case 5.1 Å. and for the latter 5.2 Å.

**The Dissociation of Some Calcium Salts.** BY ISIDOR GREENWALD. *From the Department of Chemistry, New York University College of Medicine, New York*

From the solubility of calcium sulfate, calcium carbonate, and calcium phosphate in solutions containing organic anions, it appears that the calcium salts of many organic acids are not completely dissociated, even in high dilution. This is confirmed by a lowering of the pH of solutions containing mixtures of the sodium salt and acid upon the addition of  $\text{CaCl}_2$ . The salts of dicarboxylic and tricarboxylic acids are dissociated less than are those of monobasic acids. The presence of a hydroxyl group also diminishes the dissociation. Calcium sulfate is also only partially dissociated. It is believed that the same partial dissociation of the salts of calcium, or of other alkaline earths, and consequent decrease of the apparent pK of the acid in the presence of  $\text{Ca}^{++}$

\* Edsall, J. T., and Wyman, J., Jr., *J. Am. Chem. Soc.*, **57**, 1964 (1935).

or  $\text{Mg}^{++}$ , applies also to phosphoric acid. The stability of  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  is believed to be incompatible with the view that it is the  $\text{NH}_4$  salt of an acid whose  $\text{pK}$  is 12.0.

The apparent solubility product of  $\text{Ca}_3(\text{PO}_4)_2$  is increased 6-fold by the change from mM fumarate to malate and 2000-fold by its change to maleate. Similarly, in mM solution, the change from ascorbate to diketogulonate markedly lowers the apparent solubility product and it is, even then, several times that in the control.

Both glycerophosphates markedly increase the solubility of calcium sulfate and carbonate. Calcium phosphate both adsorbs glycerophosphate and catalyzes its decomposition.

**A Toxic Effect of Sodium Hippurate.** BY WENDELL H. GRIFFITH.

*From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

The oral or intraperitoneal administration of 50 to 150 mg. of sodium hippurate in 0.1, 0.15, or 0.31 M solution resulted in a 2–4° decrease in the body temperature of 50 to 60 gm. rats. The temperature was not significantly affected by the administration of similar volumes of water, 0.5 per cent glucose solution, or 0.154 M solutions of sodium chloride, sodium bicarbonate, and sodium succinate. This effect of hippurate was temporary and disappeared within 6 to 8 hours. The effect of sodium benzoate on the body temperature was more marked and of longer duration. Comparison of the effects of hippurate and benzoate was complicated by the differences in the rates of absorption and excretion. Hippurate was found to be absorbed slowly from the alimentary tract and to be excreted rapidly by the kidneys. Benzoate was rapidly absorbed from the alimentary tract and slowly excreted by the kidneys. Both were absorbed rapidly from the peritoneal cavity. It was possible to prolong the period of subnormal temperature by the repeated injection of hippurate. The effect of benzoate and hippurate on body temperature is suggestive, in view of the recent observation that both of these substances inhibited *in vitro* the oxygen uptake of minced tissue and of tissue slices of the rat.

**Studies of Diphosphoglyceric Acid in Blood Cells.** BY GEORGE MARTIN GUEST AND S. RAPOPORT. *From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati*

Diphosphoglyceric acid in the blood cells appears to constitute a considerable labile phosphorus reserve, to serve as an important means of transport in the phosphorus metabolism of the body, and to play an important rôle in the acid-base equilibrium of the cells. In normal human, rabbit, and dog bloods, the diphosphoglycerate fraction makes up from 45 to 55 per cent of the total organic acid-soluble phosphorus. In several conditions where large changes in the organic acid-soluble phosphorus (ester P) of blood cells have been observed, such changes have been completely accounted for by changes in the diphosphoglycerate phosphorus: *increases*, following overdosage of rabbits with irradiated ergosterol, after suppression of renal function, and after pyloric obstruction and vomiting; *decreases*, in chloride and other types of acidosis. Example: In a rabbit, following the administration of 0.8 cc. of irradiated ergosterol in oil, 10,000 X, by mouth, the organic acid-soluble P in the cells increased from 95 to 119 mg. per cent, while the diphosphoglycerate P in the cells increased from 46 to 70 mg. per cent. In chloride acidosis and in pyloric obstruction, the changes in amounts of base bound by diphosphoglycerate in the cells (decreased or increased) have been found to balance closely the amounts of base not accounted for by changes in determined anions and cations, total base, Cl, CO<sub>2</sub>, hemoglobin.

**The Formation of Pyrrole by the Dry Distillation of Proteins.**

BY GORDON H. GUEST AND WILLIAM D. McFARLANE. *From the Department of Chemistry, Macdonald College, McGill University, Quebec, Canada*

Fromm's method\* for the determination of pyrrole has been modified as follows: Transfer 1 ml. of the pyrrole solution (containing 5 to 30 micrograms of pyrrole per ml.) to a 10 ml. glass-stoppered, graduated cylinder and add 0.2 ml. of a 0.5 per cent

\* Fromm, F., *Mikrochemie*, 17, 141 (1935).

solution of isatin in glacial acetic acid and 1 ml. of concentrated hydrochloric acid. After 5 minutes dilute to 10 ml. with 95 per cent alcohol. The pyrrole blue color is measured with an Evelyn photoelectric colorimeter, with the No. 660 light filter. The reaction conforms to Beer's law. 2,5-Dimethylpyrrole, 2-carbethoxypyrrole, pyrrolidine, proline, hydroxyproline, and tryptophane fail to react but 1-carbethoxypyrrole does react.

Apparently the pyrrole obtained from gelatin is derived entirely from the proline and hydroxyproline. This is confirmed by the results of fractional analysis of gelatin hydrolysates and by the fact that on the dry distillation of each of the crystalline amino acids known to be present in gelatin only proline and hydroxyproline gave pyrrole.

The percentage yield of pyrrole on the dry distillation of various substances was obtained by collecting the vapors in 5 per cent acetic acid and determining the pyrrole content of the distillate. From their proline and hydroxyproline content, and the yield of pyrrole obtained by the dry distillation of these amino acids, we can account for the amount of pyrrole obtained from a number of proteins.

**On the Determination of Glycogen in Muscle.** BY M. MASON GUEST. *From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York*

When the primary interest in a chemical analysis is centered in the "active" constituents of an organ, as in the analysis of muscle for glycogen, the greater portion of the connective tissue may be removed if the cake resulting from Graesser's method for crushing frozen tissue is broken up and passed through a suitable screen. It is necessary that provision be made for keeping the material frozen through the entire process.

The portion which passed through the screen gave an average of 10 per cent more glycogen than unscreened samples and about double that which remained on the screen. The accuracy and precision was further increased by weighing the samples just over the dew point and by protecting the samples through the processing from the deposition of water by condensation.

The probable error after all of these precautions was under 2 per cent of the mean.

**The Gonadotropic Hormone of Pregnancy Urine.** BY SAMUEL GURIN, C. BACHMAN, AND D. WRIGHT WILSON. *From the Departments of Physiological Chemistry and Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia*

Adsorption of gonadotropic hormone from early pregnancy urine upon benzoic acid, followed by washing with acetone and elution of the resulting product with 30 per cent aqueous acetone, yields preparations ranging in activity from 500 to 2000 rabbit-kilo-ovulating units per mg. A study has been made of various factors influencing the recovery of the hormone as well as the potency of these preparations. Negligible further purification is afforded by fractionation with non-aqueous solvents or partial salting-out procedures.

Preparations obtained in this manner contain approximately 10 per cent ash. They contain carbohydrate, acetyl, hexosamine, and a polypeptide complex. With different fractions the molar ratios of reducing sugar to hexosamine ranged from 5.4 to 6.4, while the acetyl to hexosamine ratios were approximately 2. The amino group of the hexosamine appears to be combined with acetyl; the position of the other acetyl is at present unknown.

The analyses, along with qualitative tests and solubility properties, indicate that the material in these hormone preparations is largely mucoid in character.

**Physiological Carcinogenesis.** BY G. E. HALL AND W. R. FRANKS. *From the Department of Medical Research, Banting Institute, University of Toronto, Toronto, Canada*

In the course of experiments to study the effect of prolonged administration of acetylcholine on various parasympathetically controlled organs (Hall, Ettinger, and Banting, 1936) an inordinate number of tumors have developed in various animals. Most are definitely malignant, some carcinomatous. None occurred at the site of the prolonged daily acetylcholine injections. Of three dogs on scrap diet surviving over 150 daily intravenous injections of acetylcholine, two died with experimentally produced myocardial disease and one developed at 161 days an osteogenic sarcoma from which it died 4 months later; the sarcoma proved transplantable. Of twenty stock rats fed Dog Chow surviving



6 months of daily subcutaneous injections of acetylcholine six have died with tumor at 11 months. Of six guinea pigs fed carrot and Rabbit Chow, surviving over 4 months of daily acetylcholine, two have developed osteogenic tumors. Of seven fowls surviving 8 months of acetylcholine, one has developed an osteogenic tumor of the tibia. Of twelve stock mice fed Dog Chow, surviving 2 months of acetylcholine thrice weekly, two died with mesoblastic tumors in the 3rd month.

The adrenals, when examined, showed evidence of (compensatory) exhaustion with beginning degenerative change. Simultaneous cortin administration should indicate whether this endogenous carcinogenesis arises from excessive (compensatory) cortical secretion or from abnormal exhaustion products.

**Detection and Estimation of Methanol, with Results in Human Cases of Methanol Poisoning.** BY R. N. HARGER, STEVEN L. JOHNSON, AND E. G. BRIDWELL. *From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis*

The presence of about 1 per cent of ethanol greatly increases the color intensity and the sensitivity of the Wright-Elvove\* reaction for methanol, ethanol alone giving no color. This modification makes it possible to run the test directly upon spinal fluid and tungstic acid blood filtrate from cases of methanol poisoning, since these fluids contain no interfering substances and the concentrations of methanol are easily within the limits of the test. Urine may also be tested directly except where the subject is receiving methenamine or large quantities of iodides. The modified method gives satisfactory quantitative results with spinal fluid, tungstic acid blood filtrate, and distillates from other body fluids and tissues. To test the accuracy of the method and see whether other alcohols were present, the results were checked against those obtained by our microdichromate method.†

Two other quantitative procedures for methanol were developed: (a) The methanol is oxidized at 100° by dichromate in the presence of 62 per cent sulfuric acid, which converts the methanol quantitatively to CO<sub>2</sub>, the latter being aerated off and absorbed

\* Wright, L. O., *J. Ind. and Eng. Chem.*, **19**, 750 (1927).

† Harger, R. N., *J. Lab. and Clin. Med.*, **20**, 746 (1935).

with ascarite. Ethanol, if present, is quantitatively converted to acetic acid, which may be distilled off and titrated. (b) The methanol is dissolved in 53 per cent sulfuric acid and titrated into a solution made by mixing 10 volumes of 53 per cent sulfuric acid with 1 volume of 0.05 N permanganate. The end-point is reached when the last trace of purple color disappears, leaving a faint brownish yellow solution. In this reaction the methanol is converted to formaldehyde. This reagent may also be used for ethanol and for determining ethanol or methanol in breath. The reaction occurs at room temperature.

By these methods, methanol determinations were made upon blood, spinal fluid, urine, and body tissues from nine human cases of methanol poisoning. The concentrations of blood methanol in six fatal cases ranged from 1.14 to 2.38 mg. per cc. The distribution of methanol in the body was very similar to that which we have previously found for ethanol.\*

**The Effect of Physostigmine on the Blood Sugar of Rats with Demedullated Adrenals.** BY BEN K. HARNED AND VERSA V. COLE. *From the Laboratory of Pharmacology, Woman's Medical College of Pennsylvania, Philadelphia*

The subcutaneous injection of 0.1 mg. of physostigmine salicylate per kilo of rat produces under appropriate conditions in demedullated animals a marked hypoglycemia. The minimal blood sugar value appears 3 to 5 hours subsequent to the drug injection. Our most pronounced effects have been obtained by using the drug in conjunction with a standardized glucose tolerance test, since the phenomenon appears to depend upon the combined action of an elevated blood sugar and the physostigmine.

45 minutes subsequent to the physostigmine injection 3.5 gm. of glucose per kilo were injected intraperitoneally and blood sugar determinations were made at intervals of  $\frac{1}{2}$ , 1, 2, 3, and 5 hours. The difference between the blood sugar values obtained with and without the physostigmine served as a measure of the effect of the drug. The data were obtained on seventeen rats. In the presence of the drug the minimal blood sugar values average 30

\* Harger, R. N., Hulpieu, H. R., and Lamb, E. B., *J. Biol. Chem.*, **120**, 689 (1937).

mg. per cent less than in the control. Objectionable side reactions were not observed.

**Preparation of Sugar Acids from *d*-Glucose.\*** By J. P. HART, FAY SHEPPARD, AND MARK R. EVERETT. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

Calcium 5-ketogluconate may be prepared in improved yields from *d*-glucose or *d*-gluconolactone by bromine oxidation. 1 per cent solutions of the sugar or lactone are maintained in contact with liquid bromine until the Sumner reducing value reaches 45 per cent or more, which usually requires at least 6 weeks at 25°. The excess bromine is removed by aeration and the solution neutralized to approximately pH 6.5 with calcium carbonate and a little alkali. After concentration *in vacuo* to one-fifth of the original volume and seeding the solution, calcium 5-ketogluconate crystallizes. In this way 30 to 33 per cent yields are obtained. The product is quite pure, but may be recrystallized from water.

The mother liquors yield calcium saccharate and a small amount of another reducing calcium salt which is being investigated. The 5-ketogluconic acid is best obtained from its calcium salt by the action of sulfuric acid in anhydrous solvents. A study of the derivatives of this difficultly crystallizable acid is in progress.

The bromine oxidation method is superior to Kiliani's nitric acid method for preparing calcium 5-ketogluconate. The former gives equally good results with either glucose or gluconolactone; the latter does not. Glucose heated 2½ hours with the quantity of nitric acid calculated for complete conversion to 5-ketogluconic acid contains unchanged glucose, as shown by fermentation, and gluconolactone gives appreciable quantities of an aldehyde or other labile acid destroyed by subsequent bromine oxidation.

**Comparative Distribution in the White Rat of Radioactive Phosphorus Fed As Sodium Phosphate and Injected As Phospholipid.** By FRANCES L. HAVEN, WILLIAM F. BALE, AND MARIAN L. LEFEVRE. *From the Department of Biochemistry and Phar-*

\* Aided by a grant from the Research Appropriation of the University of Oklahoma Medical School.

*macology and the Department of Medicine, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

Phosphorus metabolism has been studied in growing and adult white rats, with artificially radioactive phosphorus as a tracer. Quantitative data are reported giving the distribution of phosphorus fed as sodium phosphate in bones and teeth, internal organs, muscle, blood, and carcinosarcoma No. 256 as well as in the phospholipids of various organs. A large portion of the phosphorus is rapidly transferred to the bony structure of the animal. It is of interest that, shortly after feeding, the concentration of the administered phosphorus in the heart is nearly 10 times that in skeletal muscle, and several times that of blood.

The concentration of administered phosphorus rises rapidly in tumor tissue and in its phospholipid fraction, the concentration in tumor phospholipid, a few hours after feeding, being of the same order as in the phospholipid of liver and kidney and many times that of muscle.

Preliminary results are also reported on the distribution of radioactive phosphorus injected as phospholipid.

**Changes of Nitrogen Content Brought About by Denaturation of Proteins.** BY BYRON M. HENDRIX AND JOE DENNIS. *From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston*

221 determinations of the nitrogen content of three different samples of crystalline egg albumin gave a value of  $15.38 \pm 0.04$  per cent. Portions of these samples were denatured by acid treatment, by alkali treatment, and by shaking. The acid-denatured albumin contained  $14.93 \pm 0.03$  per cent nitrogen (87 determinations). The alkali-denatured albumin contained  $15.09 \pm 0.04$  per cent nitrogen (222 determinations), and the albumin denatured by shaking  $15.05 \pm 0.04$  per cent (111 determinations). A sample of crystalline edestin was analyzed and found to contain  $18.70 \pm 0.04$  per cent nitrogen (100 determinations). A portion of this sample was denatured by long contact with water. The nitrogen content of the denatured product was  $18.55 \pm 0.03$  per cent (100 determinations). All nitrogen values are on a moisture- and ash-free basis. In all cases the denatured products had a lower nitrogen content than the corresponding crystalline materials.

Analysis of the filtrates in which the denaturations were carried out revealed that the nitrogen split off during the denaturation was entirely inadequate to account for the observed lowering. The only available explanation for these results is that the protein has taken up water during the denaturation process. When correction is made for the nitrogen split out of the edestin molecule, the denatured protein is found to consist of 99.3 per cent of the crystalline protein and 0.7 per cent water. The alkali-denatured albumin has taken up 1.5 per cent water, the acid-treated albumin 2 per cent, and the albumin denatured by shaking 2.7 per cent. The crystalline albumin represents 98.5 per cent, 98 per cent, and 97.3 per cent respectively of these denatured albumins.

**Secretion of Ingested Sulfanilamide in Human Milk and in the Urine of the Infant.** BY JOSEPH S. HEPBURN, NEWLIN F. PAXSON, AND ALAN N. ROGERS. *From the Departments of Chemistry and Obstetrics, the Hahnemann Medical College and Hospital of Philadelphia, Philadelphia*

Study was made of the occurrence of sulfanilamide (*p*-aminobenzenesulfonamide) in the milk of lactating women for whom that compound had been prescribed. The women were in the puerperium. The daily dose of sulfanilamide was 25 grains, in divided doses of 5 grains by mouth at intervals of 4 hours. In sixteen cases, milk was secured for analysis after medication for 24 hours and was tested for sulfanilamide. With two of the patients, who continued to receive the drug for an additional period of 48 hours, additional tests were made on the milk at the end of 48, 72, 120, and 144 hours. Since the prime problem was to ascertain whether ingested sulfanilamide appears in the breast milk, the tests and determinations were limited to preformed or free sulfanilamide. In all sixteen cases, sulfanilamide was present in the milk 24 hours after beginning medication with that compound, and had a concentration between 0.55 and 2.17 mg. per 100 cc. In each of the two patients who received the medication for a total period of 72 hours, the concentration of the sulfanilamide in the milk did not increase after the 1st day, and the drug was still present in the milk to the extent of 0.23 to 0.30 mg. per 100 cc. at the end of 144 hours; *i.e.*, 72 hours after medication had ceased.

In six experiments, the mothers received the sulfanilamide in the stated dosage for several additional days, and the urine of each infant was tested for the presence of free sulfanilamide 24 hours after beginning medication of the mother. It was present in a concentration of less than 0.1 mg. per 100 cc. of urine in each case. Analyses were also made on the urine of several of the infants for 3 additional days; the sulfanilamide concentration in these specimens ranged between 0.19 and 1.40 mg. per 100 cc. of urine.

**Cystinuria; the Effect of Feeding Methionine and Cysteine on the Excretion of Cystine.** BY W. C. HESS AND M. X. SULLIVAN.  
*From the Chemo-Medical Research Institute, Georgetown University, Washington*

Cystine studies were made on a cystinuric on a prescribed diet over a considerable period. Estimations were made by the Sullivan and the Okuda cystine methods of free cystine in the urine, cystine in the sediment, and cystine liberated by hydrolysis. After hydrolysis, the findings by both methods were of the same order of magnitude. Feeding methionine and cysteine to the same individual did not increase the cystine content of the urine as measured by the fore and after periods respectively. After both methionine and cysteine there was a marked increase in the inorganic sulfates.

**Preparation of Deutero Fatty Acids.** BY W. E. VAN HEYNINGEN.  
*From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

For the biological work in which deuterium is used as an indicator in metabolic studies with fatty acids of different chain lengths new methods had to be developed for the introduction of the isotope into the fatty acids. Treatment with deuteriosulfuric acid brings about the introduction of deuterium into fatty acids, from which the isotope cannot be removed by treatment with dilute acid or alkali. Closer investigation has shown that the deuterium thus introduced is attached practically completely to the  $\alpha$ -carbon atom. Such material does not seem to be suitable for all experiments, especially those involving biological degradation.

By making use of exchange reactions similar to those described

by Horrex and Polanyi it has been found possible to prepare fatty acids containing 20 to 30 atom per cent deuterium. Ordinary fatty acids are treated with  $D_2O$ , alkali, and active platinum at elevated temperatures. In the resulting acids deuterium must be attached to at least a third of the carbon atoms. On treatment successively with hot 20 per cent sulfuric acid and 7 per cent alkali the acids do not lose deuterium.

The biological application of these compounds is discussed.

**Hyperparathyroidism in Experimental Renal Insufficiency.** BY WALTER J. HIGHMAN, JR. *From the Department of Pediatrics, University of Chicago, Chicago*

Data are presented on the interrelationships of blood calcium, phosphorus, non-protein nitrogen, parathyroid function, and histological changes in the parathyroid glands in experimental renal insufficiency. This renal insufficiency was produced in dogs by removing a large percentage of kidney substance. Parathyroid hyperfunction, measured by the method of Hamilton and Highman, increased with the severity and duration of the renal damage; and the degree of rise was paralleled roughly by the changes in the parathyroid glands later found at autopsy. These changes took place whether or not the serum phosphorus was elevated, and the serum calcium remained within normal limits throughout the experimental period for each dog. There was generally no parallelism between the degree of non-protein nitrogen retention and the amount of increase in parathyroid function; nevertheless, the dog whose non-protein nitrogen reached the highest level showed the greatest secondary hyperparathyroidism functionally and anatomically.

**The Denaturation of Dried Hemoglobin by Molecular Oxygen.**

BY ALAN HISEY. *From the Department of Chemistry, University of Tennessee School of Biological Sciences, Memphis*

The gaseous uptake by dried reduced hemoglobin and dried methemoglobin at 38° has been followed in the presence of oxygen and carbon monoxide, with specially designed Warburg microrespirometers. The rate of absorption by dried reduced hemoglobin is initially more rapid but finally less complete for carbon monoxide than for oxygen. In the presence of oxygen dried

reduced hemoglobin is converted to methemoglobin, which after prolonged exposure is denatured. There is no evidence that carbon monoxide produces these effects. Dried methemoglobin takes up oxygen more slowly, and, within the period studied, about half the quantity absorbed by dried reduced hemoglobin; denaturation occurs in both cases in about the same time. Dog hemoglobin and methemoglobin are more susceptible to denaturation by molecular oxygen than either ox or human. There is no evidence that adsorption occurs or that carbon dioxide is liberated. It has been shown previously that dried reduced hemoglobin sealed *in vacuo* can be preserved without loss of activity for at least a year.\*

This evidence shows that denaturation of dried hemoglobin is brought about by the action of molecular oxygen. It is probable that specific groups in the globin molecule are being oxidized, as indicated by the species variation. This question is being further investigated.

**The Molecular Constitution of the Calcium Phosphates.** BY HAROLD CARPENTER HODGE AND WILLIAM F. BALE. *From the Department of Biochemistry and Pharmacology and the Department of Medicine, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

Electrometric titration of  $\text{Ca}(\text{OH})_2$  with phosphoric acid produces a curve in which an anomalous inflection at about pH 7.5 is sometimes found at room temperatures. The inflection becomes pronounced at 5° and disappears at 40°. Chemical and x-ray analyses of the precipitates at various points along the curve are compared for the different temperatures.

**The Fate of Glucose Solutions Introduced into the Stomach of Humans.** BY O. D. HOFFMAN, W. O. ABBOTT, WALTER G. KARR, AND T. G. MILLER. *From the Laboratory and Gastro-intestinal Section of the Medical Clinic, University of Pennsylvania Hospital, Philadelphia.*

It has been previously reported that the amount of glucose leaving the stomach is dependent on the quantity and volume

\* Morrison, D. B., and Hisey, A., *J. Biol. Chem.*, 117, 693 (1937).



introduced. This product is further correlated with the product of the volume and concentration.

By means of the three- and two-lumened tubes devised by Miller and Abbott it has been possible to obtain samples of gastric and duodenal contents simultaneously at five levels, cardia, pylorus, duodenal cap, mid-duodenum, and just proximal to the ligament of Treitz. Specimens were collected every 5 to 10 minutes for a period of 1 to 2 hours.

There is a distinct dilution gradient between the stomach, the upper, the mid-duodenum, and the lower duodenum. The specimens from the duodenal cap and mid-duodenum vary in concentration, are less than 15 per cent, vary with motility, and inversely with the time since expulsion from the pylorus. The concentration in the lower duodenum is more constant, 3 to 6 per cent.

In the stomach strong solutions are diluted continuously, often to a low concentration, but the absolute amount of glucose leaving the stomach is dependent, not on such dilution, but on the product of the volume times the concentration introduced. There is a distinct retention in the stomach of the stronger glucose-solutions, although some sugar is immediately released into the duodenum.

By blocking the intestine and removing all sugar not absorbed by the stomach and duodenum as a unit, absorption values were obtained which indicate the same relationship as that for the stomach. However, experiments with high volumes times concentration were not done.

**A Rapid Photoelectric Method for the Microdetermination of Sodium in Biological Fluids.** BY WILLIAM S. HOFFMAN AND BESS OSGOOD. *From the Department of Physiological Chemistry, Chicago Medical School, Chicago*

The sodium concentration of human serum is about  $143 \pm 5$  milli-equivalents per liter. A deviation of 4 per cent or more from the average normal concentration is probably of pathological significance. Micromethods for serum sodium, therefore, which have an error of 3 or more per cent, such as ordinary colorimetric methods, can have little value in physiological or clinical investigations. However, with the use of a photoelectric colorimeter (Cenco-Sheard-Sanford photometer), a micromethod for sodium has been developed, which, while rapid and comparatively simple has an error of only about 1 per cent.

0.2 cc. of serum is wet ashed directly in a 15 cc. Pyrex centrifuge tube and treated with zinc uranyl acetate, as in the gravimetric method of Butler and Tuthill. The yellow precipitate of sodium zinc uranyl acetate is centrifuged, washed once with a special wash liquid, and twice with ether. It is then dissolved in 10 cc. of water and read in the photometer with a blue filter. Calculations are made by reference to a curve or table prepared from known concentrations. Since the color is sensitive to temperature changes, the room temperature must be controlled, or a correction curve applied. The blank for the reagents used must be accurately determined. Urinary sodium can be similarly determined, without previous ashing, if phosphate has been removed.

### **Characteristics of Gastric Secretion Stimulated by Pilocarpine.\***

BY FRANKLIN HOLLANDER AND MAX SALTZMAN. *From the Laboratories of the Mount Sinai Hospital, New York*

Literature on gastric secretion induced by pilocarpine presents the following contradictory views: (a) the acidity is considerably lower than that of histamine secretion, because pilocarpine stimulates the production chiefly of organic constituents and only secondarily of HCl and water; (b) pilocarpine stimulates juice of high acidity, not essentially different from histamine secretion. The effect on gastric secretion of subcutaneous injection of pilocarpine was studied in dogs with Pavlov pouch. Irritation by the collecting device, and hence extraneous stimulation of mucus secretion and other contaminants, was eliminated by use of pouches of the sphincter type, previously described. Secretion was collected by either of two methods: (a) *continuous collection* with a collector in place throughout the experiment (C experiments); (b) *periodic removal* of fluid retained in the pouch by sphincter action (R experiments).

**Results**—(1) The usual responses to pilocarpine (restlessness, vomiting, etc.) were manifested, but in varying degree for any one animal. (2) Latent periods varied from 10 minutes to 1 hour, in contrast with histamine. (3) Duration and total volume of secretion varied considerably. (4) Retained juice contained little or no mucin; the other contained appreciable amounts. (5)

\* Supported in part by a grant from the Friedsam Foundation.

Traces of blood were usually obtained in the C experiments but rarely in the R experiments. (6) Total acidities were like those obtained with histamine—as high as 150 to 160 mm in the R experiments, significantly lower in the C experiments. (7) Combined acidities were consistently higher than in histamine experiments; C experiments yielded higher values than R experiments. The implication of these observations, particularly the relation of collection procedure to properties of the secretion, is discussed in detail.

**A Method for the Extraction of Cholesterol from Blood.** BY FREDERIC E. HOLMES AND GLENN E. CULLEN. *From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati*

In the Leiboff method or the determination of total cholesterol of blood by extraction of the blood which has been placed on filter paper, much difficulty has been experienced owing first to the varying amounts of water, and secondly, to the errors resulting from drying the blood on the filter paper. In the present method the extraction is modified by the introduction of a trap below the reflux condenser which removes the water from the chloroform. This results in a satisfactorily accurate method without sacrificing the advantage of simplicity. That the method is accurate is shown by comparison with the digitonide method of Van Slyke, Page, and Kirk. The method is applicable to plasma or serum as well as to blood. Strict adherence to the details of procedure involves no difficulty but is necessary to insure the agreement with the digitonide method.

**A Simple Glass Electrode for the Estimation of the pH of Biological Fluids under Anaerobic Conditions.** BY M. K. HORWITT. *From the Biochemical Research Laboratory, Elgin State Hospital, Elgin, Illinois*

The procedure for determining pH of blood, spinal fluid, etc., can be facilitated by building a glass electrode into the barrel of a hypodermic syringe, so that the determination can be made directly on the fluid in the syringe without transferring the material. By fusing Corning No. 015 glass onto the plunger of a

1.5 cc. syringe, it is possible to make immediate contact of the electrode system with the fluid under investigation. The hollow part of the plunger is used to contain the chemicals of the electrode system.

**Further Investigations of the Absorption of Minute Amounts of Lead from the Diet.** BY M. K. HORWITT AND GEORGE R. COWGILL. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

A continuation of the study of the tolerance of animals to small amounts of lead demonstrated the influence of the various constituents of the diet upon the rate of absorption of the lead in the diet. Whereas previous studies on dogs\* had indicated that the amounts of lead found in the body were related to the amounts ingested, a more recent investigation with a slightly different diet, involving analysis of the entire animals after 6 months of growth, gave no such correlation. Puppies receiving 52 mg. of lead per kilo of diet retained only slightly more lead than those animals receiving 2 mg. per kilo. The diet of these animals consisted of 87.9 per cent whole milk powder, 10 per cent Crisco, 2 per cent agar, and 0.1 per cent salt mixture,† whereas the diet used in the earlier experiments consisted of 41.2 per cent casein, 29.4 per cent sucrose, 18.3 per cent lard, 7.2 per cent butter, 2.6 per cent bone ash, and 1.3 per cent salt mixture.† It is quite possible that the milk powder in the diet prevented the absorption of lead, but on the basis of the work done one cannot yet rule out the possible importance of the agar in making the lead insoluble.

Analysis of the feces of the experimental animals on the milk powder diet showed that nearly all of the ingested lead could be accounted for in the feces.

**The Specificity of the  $\beta$ -Alanine Radical in Relation to the Depressor Action of *l*-Carnosine.** BY MADISON HUNT AND VINCENT DU VIGNEAUD. *From the Department of Biochemistry, School of Medicine, George Washington University, Washington*  
With the demonstration of the specificity of carnosine with

\* Horwitt, M. K., and Cowgill, G. R., *Proc. Soc. Exp. Biol. and Med.*, **36**, 744 (1937); *J. Pharmacol. and Exp. Therap.*, **61**, 300 (1937).

† Cowgill-Karr, see Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923).

regard to spatial configuration we have become interested in various aspects of the relation of the structure of carnosine to its effect on blood pressure. As a step in this direction we have investigated the importance of the  $\beta$ -alanyl moiety.

The first point to be established was, of course, whether the  $\beta$  position of the amino group was requisite to the depressor action. Both *d*- $\alpha$ -alanyl-*l*-histidine and *l*- $\alpha$ -alanyl-*l*-histidine were therefore synthesized. Interestingly enough, neither of these peptides possessed depressor activity.

The synthesis of various isomeric aminobutyryl peptides of histidine was next undertaken. From such a series of isomers we felt that we could again test the importance of the  $\beta$  position, since we might be able to compare the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -aminobutyryl peptides and that we could obtain evidence of whether a 3-carbon chain was necessary for the depressor action. To test the importance of a terminal amino group, a comparison of glycyl-*l*-histidine and the  $\gamma$ -aminobutyryl-*l*-histidine with carnosine seemed to offer a method of approach. Furthermore, the *d*- and *l*- $\alpha$ -methyl- $\beta$ -aminopropionyl derivatives of *l*-histidine should afford evidence with regard to the necessity of the presence of 2  $\alpha$ -hydrogen atoms in the  $\beta$ -alanyl moiety.

The peptides have been synthesized and are now being tested for depressor action. These pharmacological results will also be reported. Early indications are that the  $\beta$ -alanyl radical is amazingly specific for the depressor action of carnosine.

**Further Studies on Octopine.** BY J. LOGAN IRVIN. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

Octopine, a nitrogenous compound isolated by Morizawa from octopus muscle in 1927 and by Moore and Wilson from scallop muscle in 1934, was shown by the latter investigators to be arginine, the  $\alpha$ -amino group of which is joined to the  $\alpha$ -carbon atom of propionic acid.

Further studies upon the compound have been carried out by us with the following results. Copper, nickel, and Reinecke salts have been prepared and analyzed. The electrometric titration curve of octopine has demonstrated the presence of two acidic and two basic groups. This structure has been confirmed also

by a synthesis of partially optically inactive octopine by means of a reaction between the esters of *d*-arginine and *dl*- $\alpha$ -bromopropionic acid in an alcoholic solution. A similar synthesis was made by Akasi.

Both octopine and arginine have been isolated by us from scallop, squid, and octopus muscle. Studies carried out on fresh scallop muscle have indicated that arginine is the precursor of octopine. Arginine, which, in fresh muscle dissected from live scallops, constituted about 90 per cent of the total guanidine bases analyzable by the Sakaguchi method, was found to decrease to about 5 per cent of the total during autolysis of the scallop muscle slices for 3 days at 0°. During this period the amount of octopine was found to increase reciprocally. These results were obtained by analyses and were confirmed by isolations. The synthesis of octopine from arginine was more rapid in tissue slices than in hashed tissue.

**Kynurenic Acid Excretion by Carnivora.** BY RICHARD W. JACKSON. *From the Department of Biochemistry, Cornell University Medical College, New York City*

Several mammals have been studied by various workers with respect to excretion of kynurenic acid (see summary of results\*). Of the Carnivora, only the dog and the coyote have been reported to excrete the product, whereas the domestic cat has been shown repeatedly not to eliminate the substance. The present study deals with other members of the dog and cat families as well as representatives of other families of the Carnivora. Generally, a single carefully controlled experiment was made on any one species. The animals weighing from 0.9 to 136 kilos were given orally from 1.6 to 7 gm. of tryptophane each and the urines collected from the cages during the following 48 hours. The urines were analyzed for kynurenic acid by the procedure of Capaldi, supplemented with the sensitive color test of Jaffe. The identity of each specimen of kynurenic acid secured was confirmed by preparation of an authentic derivative. Two additional members of the cat family (*Felidae*), the cheetah and the serval, were found not to excrete

\* Gordon, W. G., Kaufman, R. E., and Jackson, R. W., *J. Biol. Chem.*, **113**, 125 (1936). Ichihara, K., and Goto, S., *Z. physiol. Chem.*, **243**, 256 (1936).

the acid. The experiments on the genet and the civet (Viverridæ), the raccoon (Procyonidæ), the brown bear (Ursidæ), and the sealion (Otariidæ) were also negative in this respect. However, all of the Canidæ examined, the coyote, the fox, and the wolf, as well as the hyena (Hyænidæ) and the badger (Mustelidæ), do excrete the compound following the ingestion of tryptophane.

This investigation was made possible by the generous cooperation of the New York Zoological Society.

**Decystinized Casein.** BY D. BREESE JONES AND CHARLES E. F. GERSDORFF. *From the Protein and Nutrition Research Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington*

When casein is dispersed in dilute NaOH and reprecipitated with acetic acid at pH 4.5, and the process repeated five or six times, a product is obtained practically devoid of cystine. The total N, the distribution of N, and percentages of arginine, histidine, lysine, tyrosine, and tryptophane differed but slightly from that of the original casein.\*

Further work has now confirmed the earlier observations on the disappearance of cystine. It has also revealed a drop in the total S content from 0.83 per cent in the casein to 0.53 per cent in the decystinized product, and a decrease in the P content from 1.11 per cent to 0.51 per cent. The methionine value remained unchanged.

Striking changes in physical properties also occur. Untreated casein is not dispersed in alcohol at different concentrations and temperatures, and undergoes little or no change in appearance. In 30 per cent alcohol at room temperature decystinized casein soon becomes translucent and sticky. It is completely dispersed in boiling 30 per cent alcohol, but somewhat less in boiling 60 per cent alcohol, and not dispersed at all in absolute alcohol. On cooling from hot alcohol, it separates and settles as a soft, sticky precipitate. The decystinized product does not differ significantly from casein in digestibility *in vitro*, but it has a lower "true protein" value as determined by the Stutzer method. Studies are in progress to determine its biological value. A protein devoid of cystine and

\* Jones, D. B., and Gersdorff, C. E. F., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **99**, p. civ (1934).

nutritionally adequate would be valuable for use in the rations of experimental animals used in certain types of nutritional and metabolism studies.

**The Simultaneous Microdetermination of the Total Base and Chloride of Serum by Electro dialysis.** BY NORMAN R. JOSEPH AND WILLIAM C. STADIE. *From the Laboratories of the Department of Research Medicine, University of Pennsylvania, Philadelphia*

The Keys-Adair apparatus for the determination of total base in serum was modified by the addition of an anode chamber in which a platinum electrode is immersed. By using a dilute solution of glucose as a reducing agent in the anode chamber, oxidation of the chloride ion to free chlorine is prevented and thus loss of chloride is avoided. Acetic acid in the anode chamber serves to transport the current. The total chloride free of protein collects in the anode chamber and is determined by the Volhard method. The total base simultaneously collects in the cathode chamber and is determined as in the Keys-Adair method. Excellent recoveries of both chloride and base were obtained with 0.5 ml. of serum, with standard methods as reference.

**The Antiparalytic Vitamin of the Chick.** BY THOMAS H. JUKES AND SIDNEY H. BABCOCK, JR. *From the College of Agriculture, University of California, Davis*

A basal diet for chicks, previously described,\* produced a nutritional paralysis which was prevented by addition of the unsaponifiable fraction of soy bean oil to the diet. The same diet was used in the present investigation. From twelve to twenty birds were used in a group. The addition of 10 per cent of alfalfa meal to the diet was found to promote growth and to protect against paralytic symptoms. 10 per cent of hexane-extracted alfalfa meal was similarly effective, but the hexane extract, fed at a level equivalent to 40 per cent of alfalfa meal, had only a very slight effect. Most of the potency was removed from the hexane-extracted alfalfa meal by two extractions with warm water. The water extract was concentrated and fed at a level equivalent to

\* Babcock, S. H., Jr., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, **36**, 720 (1937).



10 per cent of alfalfa meal, and appeared to be as effective as the original material.

The addition of 0.015 per cent of nicotinic acid amide to the basal diet brought about no improvement, and resulted in excessive early mortality. Peanut meal, fed at a 15 per cent level, was only partially effective.

The symptoms were not produced in chicks by feeding a diet of natural feedstuffs which had been treated with a solution of ferric chloride in ether.

**A Spectroscopic Study of Isomerism in the Fatty Acids.** BY J. P. KASS, E. S. MILLER, AND GEORGE O. BURR. *From the Department of Botany, University of Minnesota, Minneapolis*

The photoelectric spectrophotometer of high sensitivity has been described elsewhere. The straight chain saturated fatty acids have a mean molecular extinction coefficient of 19 at 2300 Å. The unsaturated acids have an absorption of similar magnitude when not conjugated. Conjugation increases this value several hundred times, while the position of the band depends upon the number and position of the conjugated systems. The spectroscopic method has been compared with the diene procedure of Diels and Alder, which is shown to be greatly affected by oxidation products, hydroxyl groups, etc. The degree of isomerization in fats has been measured quantitatively and correlated with biological chemical studies. Selective hydrogenation by the animal has also been demonstrated and the rate followed.

**The Metabolism of Reduced Lactalbumin in Cystinuria.** BY BEATRICE KASELL. *From the Departments of Biological Chemistry and Urology, College of Physicians and Surgeons, Columbia University, and the Squier Urological Clinic of the Presbyterian Hospital, New York*

The metabolism of lactalbumin\* and reduced lactalbumin was studied in the cystinuric patient\* A under standard conditions (basal level somewhat higher than previously). Lactalbumin and reduced lactalbumin had the same total S and methionine contents (1.42 and 2.8 per cent respectively). Lactalbumin contained

\* Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., *J. Biol. Chem.*, **119**, 669 (1937).

3.1 per cent cystine and a trace of cysteine; reduced lactalbumin 0.53 per cent cystine and 2.5 per cent cysteine. The procedure was as described previously.\*

The excretion of extra N and extra total S resulting from the superimposition of 100 gm. of each of the two proteins was the same: 8.5 gm. of N and 0.73 gm. of S with lactalbumin; 8.7 gm. of N and 0.71 gm. of S with reduced lactalbumin. The excretion of extra cystine after reduced lactalbumin was much greater than after lactalbumin (39 and 16 per cent of the extra total S respectively). On the other hand, the excretion of extra inorganic sulfate was greater after lactalbumin than after reduced lactalbumin (81 and 48 per cent respectively).

The findings indicate that the extra cystine in the urine of the cystinuric is derived not from the cystine, but from the methionine and also from the *cysteine* in the protein. Cystine and cysteine, when fed as part of a protein molecule, are metabolized in the same way as when administered in the form of the free amino acids. The results demonstrate that under biological conditions cystine and cysteine as part of the same protein molecule behave as two different amino acids, with separate catabolic pathways.

**The Chemical Nature and Physiological Activity of Cortin and Crystalline Cortin-Like Compounds.** BY EDWARD C. KENDALL, HAROLD L. MASON, WILLARD M. HOEHN, AND BERNARD F. MCKENZIE. *From the Section on Biochemistry, The Mayo Foundation, Rochester, Minnesota*

Compounds A and B of the  $O_4$  series (B is corticosterone of Reichstein) are the full qualitative equivalent of the total extract of the adrenal cortex. This has been shown in regard to survival of adrenalectomized animals, capacity for work in Ingle's rat test, influence on carbohydrate metabolism, distribution of electrolytes in serum, excretion of sodium, potassium, and urea by the kidney, protection against toxic effects of potassium, and production of atrophy of the adrenal gland in normal rats by administration of large doses. Compounds E and F,  $C_{21}H_{30}O_6$ , of the  $O_5$  series maintained capacity for work in Ingle's rat test. After removal of these and other crystalline compounds an amorphous fraction from 60 to 100 times more active than Compound B is separated by repeated distribution between water and benzene and removal of

crystals insoluble in chloroform. Between 1 and 2 micrograms of this material per kilo of body weight per day are sufficient for adrenalectomized dogs. This fraction with periodic acid yields both acid and non-acid material. Oxidation of the acid fraction with chromic acid gave a non-acid from which a small amount of Ketone 4 was isolated, and a second acid fraction. Oxidation of the non-acid fraction gave principally a non-acid and some acid which yielded a small amount of Acid 1.

**The Stability of Hydrogen and Deuterium in Amino Acids.** By ALBERT S. KESTON AND D. RITTENBERG. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

For the use of deuterium as a tool for amino acid metabolism knowledge of the stability of the various hydrogen atoms in them under conditions of protein hydrolysis is of greatest importance. It can be investigated by two methods. (1) A deuterio compound with deuterium in known positions is treated with  $H_2O$ . (2) An ordinary amino acid is treated with  $D_2O$ . Twelve amino acids representing all types have been investigated. Amino acids contain three classes of hydrogen differing in their stability. (a) Those, such as the hydrogen in carboxyl and amino groups, which exchange very rapidly on mere solution in water. These are of no concern for biological work, since they bear no relation to metabolic processes. (b) Another group comprising almost all of the other hydrogen atoms is completely stable and does not exchange. (c) A third group exchanges very slowly under conditions of hydrolysis. A few amino acids (glycine, glutamic acid, cystine, and tyrosine) contain such slowly exchangeable hydrogen. In tyrosine the hydrogen atoms adjacent to the phenolic group are semilabile, since on bromination the deuterium is removed. Deuterium, present in all such positions, can be removed by appropriate treatment. The experiments indicate that amino acid metabolism can be investigated if account is taken of these properties of the hydrogen atoms.

**Filtration Processes in the Extremities Resulting from Quiet Standing.** By ANCEL KEYS AND HUGH R. BUTT. *From the Departments of Physiology and Physical Education, University*

*of Minnesota, Minneapolis, and the Division of Biochemistry,  
The Mayo Foundation, Rochester, Minnesota*

In six subjects, five normal young men and one normal young woman, blood was taken from arm veins after 30 minutes in the basal fasting state. The bed was then tipped to 54° from horizontal and blood taken from arm and foot veins 12 to 35 minutes later. All samples were taken without stasis, and all determinations were made in duplicate. The following analyses were made: albumin, globulin, cholesterol, total fatty acids, total lipids, colloid osmotic pressure of serum, oxygen capacity of whole blood.

The concentration of serum proteins increased by an average amount corresponding to a loss of 21 per cent of the plasma water in the foot and a loss of 10 per cent in the arm. In blood serum from both the foot and arm the percentage changes of cholesterol, total fatty acids, and total lipids were, within analytical error, identical with the percentage change in protein concentration.

The colloid osmotic pressure increased, on the average, 54 per cent in blood serum from the foot and 28 per cent from the arm. The corresponding values for colloid osmotic pressure per gm. of serum protein showed changes of +28 per cent and +11 per cent respectively. Assuming no change in average molecular size of the proteins, changes in colloid osmotic pressure per gm. of 24 per cent and 11 per cent, respectively, were predicted from dilution curves for colloid osmotic pressure *versus* concentration.

It is concluded that filtration in the extremities in quiet standing involves water and crystalloids only and that albumins, globulins, total lipids, cholesterol, and total fatty acids are all restrained quantitatively to the same extent by the capillary walls.

**Polyneuritis As a Criterion in Vitamin B<sub>1</sub> Determinations.** By  
O. L. KLINE, CHESTER D. TOLLE, AND E. M. NELSON. *From  
the Food and Drug Administration, United States Department  
of Agriculture, Washington*

The response of polyneuritic rats to single curative doses of crystalline vitamin B<sub>1</sub> ranging in quantity from 1 to 50 micrograms has been determined. The data obtained provided a basis for using the length of the curative period as a measure of vitamin B<sub>1</sub> content of a single dose. Evidence has been obtained indicating that the basal diet is deficient only in vitamin B<sub>1</sub>, since poly-

neuritis can be produced repeatedly in the same animal without interfering with subsequent growth and reproduction when adequate vitamin B<sub>1</sub> is provided.

**Cholesterol Content of Skin, Blood, and Tumor Tissue in Rats Irradiated with Ultraviolet Light.** BY ARTHUR KNUDSON, STUART STURGES, AND W. RAY BRYAN. *From the Departments of Biochemistry and Pathology, Albany Medical College, Albany*

Recent investigations by Roffo, Beard, and others, have shown that irradiation of rats with ultraviolet light over a long period of time produces malignant tumors on ears, nose, eyes, or other exposed parts. We have verified these observations and carried out some studies on the cholesterol content of the skin from the face, back, and abdomen of irradiated rats. We have also determined the cholesterol content of the tumors and the blood of these animals.

Our studies have indicated that the total cholesterol content of the skin is increased by irradiation, and this is particularly true in the skin from the face which showed increases in some cases of several hundred per cent and averaged about 100 per cent. The skin from the back showed an average increase of about 20 per cent, and from the abdomen only about 5 to 10 per cent. These increases of total cholesterol in the skin are practically all in the ester form.

The tumors from these irradiated animals are also very high in cholesterol and run somewhat parallel with the cholesterol content of the skin from the face of the irradiated animals. However, the free cholesterol in the tumors averages about 80 per cent, while the free cholesterol from the skin of the face of irradiated rats is 30 per cent.

A few determinations on the blood of irradiated rats show that the total cholesterol and free cholesterol content is on the average somewhat below normal. This is in striking contrast to the increases of cholesterol in both skin and tumors of irradiated animals.

**Comparison of the Oral and Intravenous Glucose Tolerance.**

BY ALFRED E. KOEHLER AND ELSIE HILL. *From the Sansum Clinic and the Santa Barbara Cottage Hospital, Santa Barbara*

The blood sugar response was determined in a series of cases after the administration of 100 gm. of glucose both orally and intravenously. The glucose was injected intravenously over a period of 2 hours as a 50 per cent solution from a 200 cc. syringe actuated by a constant speed motor. The subjects were kept on the same diet before each test and a period of 6 to 8 days elapsed between tests.

In normal individuals the blood sugar curves during and after intravenously injected and orally administered glucose bore a fairly constant ratio to each other, although there was a greater variation from the average after oral administration than during and after intravenous injection.

In a series of cases with functional gastrointestinal disturbances (hypermotility, flatulency, diarrhea), the ratio of the two curves was frequently markedly variable. It was not unusual for oral administration to be followed by a completely flat blood sugar curve (which might be interpreted as a hyperinsulin response), but the sugar curve after intravenous injection was normal or even of a diabetic nature. Such discrepancies must be explained on the basis of failure of absorption of glucose from the intestine at a normal rate.

It is concluded that variation in the absorption factor in the oral test may completely mask the utilization factor and consequently invalidate any conclusion as to the true sugar tolerance. Much greater accuracy can be obtained in detecting earlier variation of the glucose tolerance in disorders such as diabetes mellitus by the use of the intravenous method described.

**Coenzyme Stability in *Hemophilus parainfluenzæ*.** BY HENRY I. KOHN. *From the Department of Physiology and Pharmacology, Duke University Medical School, Durham, North Carolina*  
Lwoff and Lwoff\* showed that *Hemophilus parainfluenzæ* cannot synthesize either Coenzyme I or II, although it can transform one into the other, and hence these must be added to the culture medium as growth factors. During growth Coenzymes I and II are used up. Advantage was taken of this fact to estimate

\* Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London, Series B*, **122**, 360 (1937).

the minimum amount of work a coenzyme molecule can do before it is destroyed. Bacteria were grown in proteose-peptone broth to which a small amount of coenzyme was added, and after 15 to 20 hours were washed and resuspended in 0.1 M, pH 7.5, phosphate buffer. Under these conditions the oxidation of glucose, followed manometrically at 37.5°, is limited by the coenzyme content of the bacteria. The difference in the oxygen uptake in the presence and absence of added coenzyme indicates that a coenzyme molecule can be reduced (and subsequently oxidized) not less than 40,000 times.

**The Acidic Dissociation of Substituted Phenols in Relation to the Mechanism of Their Physiological Action.** BY M. E. KRAHL.  
*From the Lilly Research Laboratories, Indianapolis*

Methods recently developed in this laboratory make it possible to calculate the probable concentrations of substituted phenol anions and substituted phenol molecules in the cytoplasm of fertilized *Arbacia* eggs and other living cells which have been treated with these agents, and to follow the physiological effects of the substituted phenols in relation to such concentrations.

With any given substituted phenol, stimulation of respiration in concentrations below that for respiratory optimum appears dependent on the cytoplasmic concentration of substituted phenol anions.

The absolute cytoplasmic concentration of substituted phenol anions at the respiratory optimum was found to vary from  $1 \times 10^{-7}$  M for 2,4-dinitro-*o*-cyclohexylphenol up to  $2 \times 10^{-4}$  M for 2,4-dinitro-*o*-isopropylphenol. For such small concentrations to stimulate living cells to several times their normal oxygen uptake, it would appear that the substituted phenols must either act as catalysts themselves or profoundly affect the activity of normal cellular catalysts.

Since studies from this laboratory and elsewhere indicate that substituted phenols are unable to function catalytically, at least in cell-free systems, it may be tentatively suggested that substituted phenols stimulate cell respiration by virtue of the tendency of certain substituted phenol anions to become adsorbed on one or more of the respiratory catalysts, especially those which are believed to exist as cations in the physiological pH range. Such a

combination with the substituted phenol anion may displace the active catalyst from its normal relation to the cell structure and thus produce heightened activity. Preliminary experiments with model systems support this hypothesis.

**Further Studies of the Calcium Content of the Body As Influenced by That of the Food.** BY CAROLINE SHERMAN LANFORD AND H. C. SHERMAN. *From the Department of Chemistry, Columbia University, New York*

Rats of the same hereditary strain and of second or later generation on diets differing significantly only in calcium or calcium and phosphate content, were analyzed for total body calcium at different ages. Both the amounts and the percentages of calcium in the body were decidedly higher in the animals from diets of liberal calcium content than in those from diets of slightly above the minimal adequate level. At the age of 2 months these differences were 50 per cent or greater. The variations, probable errors, and "critical" (or "significance") ratios of the data presented are discussed.

**The Metabolism of *l*-Xylulose.** BY HARDY W. LARSON, N. R. BLATHERWICK, PHOEBE J. BRADSHAW, MARY E. EWING, AND SUSAN D. SAWYER. *From the Biochemical Laboratory of the Metropolitan Life Insurance Company, New York*

*l*-Xylulose, the sugar occurring in pentosuria, was synthesized from *d*-sorbitol. 2 cc. of a 25 per cent solution of the pentose were given by stomach tube to rats which had fasted 24 hours. After a 3 hour absorption period the animals were anesthetized, and the tissues frozen *in situ* with CO<sub>2</sub> snow and then transferred to liquid air. Glycogen, lactic acid, and fermentable and non-fermentable reducing substances were determined on liver and muscle; lactic acid, and fermentable and non-fermentable reducing substances on blood. Although we have shown that *d*-xylulose is utilized by the rat, there is no evidence that *l*-xylulose is utilized, no significant changes occurring in values for glycogen, lactic acid, fermentable and non-fermentable reducing substances. The rate of absorption of *l*-xylulose from the gastrointestinal tract is only one-third that of *d*-xylulose, and about one-half that of *d*-xylose. The coefficient of absorption of *l*-xylulose for female rats is double that for males.



**Some Observations on the Oxidation of Cysteine and Its Intermediate Oxidation Products in Aqueous Solution.** By THEODORE F. LAVINE. *From the Lankenau Hospital Research Institute, Philadelphia*

A qualitative comparison of the rates of oxidation *in vitro* of cysteine, cystine, cystine disulfoxide, and cysteine sulfinic acid sheds some light on the path of oxidation to cysteic acid. Oxidation by means of iodine in 0.05 N HCl proceeds with decreasing speed for the sulfinic acid, cystine disulfoxide, cysteine, and cystine, the latter two proceeding at about the same rate after allowance is made for oxidation of cysteine to cystine. Potassium iodate in N HCl oxidizes cystine and the sulfinic acid to cysteic acid almost immediately, while cysteine and the disulfoxide require about an hour. Potassium iodate in N H<sub>2</sub>SO<sub>4</sub> requires several hours for oxidation of cysteine and the disulfoxide, while cystine and the sulfinic acid are again oxidized very rapidly. The resistant intermediate is conjectured to be an isomeric sulfinic acid R—S(O)<sub>2</sub>H in contrast to R—S(O)OH. Decomposition of the disulfoxide in acid solution yields a product resistant to oxidation by I<sub>2</sub>, whereas decomposition in alkaline solution results in only cystine and the sulfinic acid.

Crystalline sulfinic acid is prepared in 70 to 80 per cent yield by decomposing the disulfoxide with methylamine, evaporating, filtering off cystine, adding HCl, and precipitating the sulfinic acid with alcohol.

**Dipeptidase Distribution in the Cephalic Region of the Three Day Chick Embryo.** By MILTON LEVY AND A. H. PALMER. *From the Department of Chemistry, New York University College of Medicine, New York*

Dissected 3 day embryos of chicks were properly mounted on a freezing microtome. A photomicrograph of the freshly cut surface was made and the following section used for estimation of dipeptidase by the Linderstrøm-Lang and Holter method. The activity measured by the constant  $k$  was converted to dipeptidase units ( $U$ ) as described by Palmer and Levy below. From projection prints the areas of ectoderm ( $E$ ), mesenchyme ( $M$ ), and cephalic fluid ( $C$ ) were measured. The volumes (area  $\times$  thickness) were correlated with  $U$  by the equation  $U = f_1E + f_2M + f_3C$ . The  $f$

factors represent the characteristic concentrations of dipeptidase in units per c.mm. in each kind of tissue. Gauss' method was used in solving for  $f_1$ ,  $f_2$ , and  $f_3$  in the twenty to thirty equations from a single embryo. When these factors were used to find a calculated activity, comparison with the observed activity gave a standard deviation of  $\pm 0.1$  unit in an average activity of about 0.7 unit. The averaged factors for thirteen embryo heads are  $f_1$   $10.2 \pm 2.6$ ;  $f_2$   $3.1 \pm 1.1$ ;  $f_3$   $-0.7 \pm 0.9$ . Direct studies of cephalic fluid showed that it has neither activity nor an inhibitory effect. This corresponds to the finding that  $f_3$  is not significantly different from zero. As a first approximation, the dipeptidase contents of the cephalic region of the chick embryo can be estimated from the volumes of ectoderm and mesenchyme contained therein.

**The Cystine Content of Blood Plasma after Administration of Cystine and Methionine to Rabbits.** BY HOWARD B. LEWIS AND BARKER H. BROWN. *From the Laboratory of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*

Cystine was determined in ultrafiltrates of rabbit plasma by the colorimetric method of Sullivan and Hess, with the use of the Pulfrich photometer. Cystine and methionine (sodium salts) were administered orally or subcutaneously in amounts equivalent to 0.1 gm. of sulfur per kilo. Control values were obtained from fasted animals or from animals which had received an amount of glycine equivalent molecularly to the amount of the sulfur-containing amino acids administered. The ultrafiltrates of plasma after short fasts or after glycine feeding gave values ranging from 0.96 to 1.25 mg. per cent (twelve experiments). The cystine content of the plasma ultrafiltrate was greatly increased after administration of cystine, but the values approximated fasting values after 12 hours. The amount of cystine in the plasma ultrafiltrate increased, without exception, 1 to 6 hours after administration of methionine (1.83 to 4.70 mg. per cent in 3 and 6 hour periods in ten experiments). These findings are consistent with the hypothesis that cystine may be formed in the intermediary metabolism of methionine. It should be emphasized that these results have been secured by the use of a colorimetric procedure and further evidence obtained by precipitation of the cystine from the ultrafiltrates and analyses of the precipitates (e.g.,

by some procedure such as that of Roussouw and Wilken-Jorden) is desirable.

**The Effect of Exercise on the Blood Gases, pH, and Lactic Acid Content of the Blood of Normal and Schizophrenic Subjects.**

BY JOSEPH M. LOONEY. *From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester*

The gases, pH, and lactic acid were determined in the venous blood of thirty-five normal and thirty-five schizophrenic subjects before and after running for 10 minutes up and down stairs.

The mean of work performed by patients was  $690 \pm 24.6$  kilogram-meters per minute, that for the normal subjects  $812 \pm 19.5$  kilogram-meters per minute. The increase of lactic acid was  $65 \pm 4.2$  and  $56 \pm 4.7$  mg. respectively. The patients produced  $0.097 \pm 0.007$  mg. of lactic acid per kilogram-meter per minute, which was significantly higher than that of  $0.065 \pm 0.005$  mg. for the controls.

The pH as measured by the Stadie glass electrode at 38° gave the following values: for the patients, control 7.44, after exercise 7.35, at end 7.46; for normals 7.44, 7.36, 7.47. For the patients the O<sub>2</sub> values were 11.5, 15.9, and 11.2 volumes per cent and the CO<sub>2</sub> values 57.2, 41.1, and 56.3 volumes per cent. For the normal subjects the corresponding values were 10.5, 15.5, and 11.2 and 57.8, 42.5, and 56.2 volumes per cent.

The rate of disappearance of lactic acid was the same for both groups and dependent only on the blood level. Equations fitted to the curve for removal of lactic acid were  $\log L_t = 1.77 - 0.0105t$  for the patients and  $\log L_t = 1.64 - 0.0104t$  for the normal subjects.

A high negative correlation was found between lactic acid and carbon dioxide, the coefficients being  $-0.95$  for the patients and  $-0.96$  for the normal subjects. Between lactic acid and pH a similar effect was found with coefficients of  $-0.92$  for patients and  $-0.87$  for normals. The correlation coefficients between CO<sub>2</sub> and pH were also high,  $0.85$  for patients and  $0.83$  for normals.

**Nucleic Acid from Tobacco Mosaic Virus Protein.** BY HUBERT S. LORING. *From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton*

The first preparations of tobacco mosaic virus protein were isolated by a procedure involving treatment at about pH 9 and were not found to contain detectable amounts of phosphorus, whereas preparations obtained later by improved methods were found to contain phosphorus and nucleic acid. There was some question whether nucleic acid was combined with protein and was necessary for virus activity, or could be removed by dialysis at pH 8 or 9. It has now been found that this treatment does not affect the nucleic acid content, although at pH 9 about 90 per cent loss of activity results. Repeated sedimentation of virus protein from neutral solution fails to alter the nucleic acid content. It seems likely, therefore, that nucleic acid is in combination with protein and is necessary for virus activity. These results confirm those obtained by Bawden and Pirie.

The virus protein nucleic acid may be prepared free from protein in yields of about 5 per cent by hydrolysis with NaOH at 0° or by treating a virus protein solution with glacial acetic acid. The purified virus nucleic acid closely resembles yeast nucleic acid. It is insoluble in acetic acid and is readily hydrolyzed by boiling 0.5 per cent NaOH or by 1 N HCl at 60–70°. After hydrolysis, guanine and adenine were isolated in approximately equivalent amounts, and the presence of uracil and cytosine was demonstrated by the preparation of the characteristic barium salt of dialuric acid. The virus nucleic acid isolated by glacial acetic acid treatment appears to have a somewhat higher specific rotation than yeast nucleic acid.

**The Effect of Potassium Iodate on the Liver Proteins.** By JAMES MURRAY LUCK. *From the Biochemical Laboratory, Stanford University, California*

During the dialysis stage of liver protein fractionation there is an appreciable loss of protein through proteolysis. Potassium iodate (0.001 M) prevented such losses. It also reduced by one-half the quantity of salt-soluble protein extractable at pH 5.0. The nitrogen content of the protein thus extracted was less than of that extracted in the absence of iodate. Accompanying the decrease in salt-soluble protein, effected by the iodate, there was a corresponding increase in globulin II and final residue.

Added to a solution of the salt-soluble proteins at pH 5.0,

potassium iodate hastened denaturation and inhibited, but did not completely prevent, autolysis.

When added to autolyzing liver, samples of which were analyzed daily for 10 days, potassium iodate (0.0025 M) decreased substantially the values obtained for amino nitrogen, tyrosine, total salt-soluble protein, and albumin. Globulin II was increased. The autolytic liberation of amino acids, either in the presence or absence of iodate, was at the expense of euglobulin and pseudoglobulin.

At pH 7.3 autolysis proceeded more slowly than at pH 5.0 and was almost completely inhibited by added iodate.

A tyrosine-destroying enzyme, active at pH 7.3 and inactive at pH 5.0, was found to be present.

**The Blood Plasma Cholesterol and Phospholipid Phosphorus in Control and Partially Nephrectomized Rats.** BY STEPHAN LUDEWIG. *From the Biochemical Laboratory, University of Virginia, University*

The effect of feeding diets varying in their protein and cholesterol concentrations on the plasma cholesterol and phospholipid phosphorus was determined in control and partially nephrectomized rats. It was found that renal insufficiency affected the free cholesterol values as manifested by increased plasma concentrations. No relationship was evidenced between plasma cholesterol concentrations and renal function or blood pressures. The phospholipid phosphorus varied directly with the free cholesterol concentration.

**Pancreatic Antagonism and Synergism to Insulin.** BY A. BRUCE MACALLUM. *From the Department of Biochemistry, University of Western Ontario, London, Canada*

The insulin antagonist previously reported\* as a casual constituent in duodenal preparations arises in the pancreas and is consistently found in aqueous acid and acid alcohol extracts of fasting beef pancreas.

Acid hydrolysis of duodenal preparations sometimes destroyed the antagonism and yielded preparations which increased the

\* Macallum, A. B., *Nature*, **136**, 2 (1935); *Univ. Western Ontario Med. J.*, **6**, 2

sugar tolerance of normal rabbits. Similar results occur in pancreatic preparations, the loss of antagonism being coincidental with the unmasking of a factor synergistic to the hypoglycemic action of insulin in the normal rabbit.

The synergistic fraction is soluble in acetone and can be extracted from fresh pancreatic material by this agency. The antagonist can subsequently be removed from the residual tissue by ether.

The antagonist decreases sugar tolerance and will often completely neutralize the effect of 1.5 units of insulin per kilo in rabbits. It is not adrenalin and once the physiological action is established it may persist for some weeks after withdrawal. The synergist increases the sugar tolerance and markedly accelerates the fall in blood sugar in the early phases of insulin action and prolongs the hypoglycemic action for several hours longer than do controls.

The duodenal and pancreatic antagonists are identical. The relationship between the pancreatic synergist and the insulotropic element in duodenal extracts has not been definitely established but the evidence at present indicates a pancreatic origin of the latter principle.

**The Creatine Content of Cardiac and Voluntary Muscle and Its Relation to Phosphorus and Potassium.\*** BY GEORGE MANGUN AND VICTOR C. MYERS. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

Recent studies on heart muscle have shown that creatine,† phosphorus, and potassium‡ are decreased in myocardial insufficiency. Since creatine and phosphorus are combined in living muscle as the strongly acid phosphocreatine, it appears logical to assume this phosphocreatine exists as the dipotassium salt. Its breakdown would yield 1 mole each of creatine and phosphorus and 2 moles of potassium.

Muscle from the left and right ventricles and pectoralis major were obtained from 91 autopsies. The creatine content of the left and right ventricles and pectoralis major averaged 199, 156,

\* Aided by a grant from the Josiah Macy, Jr., Foundation.

† Linegar, C. R., Frost, T. T., and Myers, V. C., *Arch. Int. Med.*, **61**, 430 (1938).

‡ Wilkins, W. E., and Cullen, G. E., *J. Clin. Inv.*, **12**, 1063 (1933).

and 408, phosphorus 188, 150, and 193, potassium 269, 206, and 315 mg. per 100 gm. of fresh tissue, respectively. These values are about 5 per cent lower than in nine cases of accidental death.

The creatine, phosphorus, and potassium averages for the left ventricle in seventeen cases of myocardial insufficiency were 26.6, 10.1, and 13.8 per cent lower, a loss of 4, 5.9, and 9.5 moles respectively. This suggests a loss of dipotassium phosphocreatine, and a small amount of some other potassium salt of phosphoric acid. Burns and Cruickshank\* have shown that adenylypyrophosphate breaks down somewhat later than phosphocreatine. This may possibly account for the additional phosphorus and potassium lost. High values for all three constituents were encountered in nitrogen retention, although with cardiac complications the results were more variable.

To aid in correctly evaluating the changes, fat and water estimations were made on a selected group of cases, as was also the acid-soluble phosphorus.

**The Toxicity of Linseed Meal.** By C. M. McCAY AND A. V. TUNISON. *From the Laboratory of Animal Nutrition, Cornell University, Ithaca*

For 50 years it has been recognized that linseed contains a toxic glycoside, linamarin. Herbivorous animals are only injured occasionally by the cyanide from this glycoside, although linseed meal is extensively used and highly prized as a feedstuff. On the other hand, it is well established that brook trout are easily poisoned by linseed meal. This is just the opposite of what might be anticipated, because the digestive enzymes in the trout work at a temperature of 8°, the gastrointestinal tract is extremely short, and food passes through it readily.

In the present study an attempt was made to learn more concerning the mechanism and the reactions within the body of the trout that lead to this poisoning from linseed meal. The hydrogen cyanide was first determined in linseed meal, after which the meal was freed from the product by such procedures as autoclaving, extraction, and acid hydrolysis. These products as well as the original meal were then fed to brook trout and the death rates recorded. Linseed meal freed from cyanide was no longer toxic.

\* Burns, W., and Cruickshank, E. W. H., *J. Physiol.*, **91**, 314 (1937).

Linseed meal fed with starch was as toxic as the original. This meal was toxic when fed with either raw or cooked liver but less so with the latter, indicating that the fresh liver promotes the toxicity but is not a primary source of enzymes for freeing the cyanide of the linseed meal.

**Blood Iodine by the McClendon-Bratton Method.** BY J. F. McCLENDON AND CARL O. RICE. WITH THE TECHNICAL ASSISTANCE OF RALPH V. WHITE AND WILLIAM C. FOSTER. *From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis*

When sodium azide was used in the absorber, the iodine could be determined on 5 cc. of blood. The time of boiling out the bromine must be reduced to a minimum and titration completed as soon as possible afterwards. With a high sensitivity galvanometer the end-point of titration is about 1000 times as sensitive as the starch iodide end-point. The average value of 60 human bloods was 0.5 microgram in 5 cc. or 10 micrograms in 100 cc.

**Inactive Androgenic Material in Human Blood and Urine.** BY D. ROY McCULLAGH, W. O. OSBORN, AND BERTHA OSGARD. *From the Department of Biochemical Research, Cleveland Clinic, Cleveland*

Inactive androgenic material has been demonstrated in urine by several groups of investigators. Different opinions have been expressed concerning the proportion of the androgens which are excreted in the inactive form. Using dibutyl ether as a solvent and less drastic but more rapid means of extraction, we have demonstrated that fresh urine contains not more than traces of free dehydroandrosterone or androsterone. The inactive androgens of urine are insoluble in chloroform, benzene, and dibutyl ether, slightly soluble in ethyl acetate, and very soluble in butanol. After the androgens have been activated by boiling with acid, they are very soluble in dibutyl ether and cannot be washed from that solvent with water or with solutions of sodium carbonate or sodium hydroxide. The amount of active androgen in the extracts was determined by bioassay with rats and capons.

In 1932 it was reported from this laboratory that androgenic materials could be demonstrated in human blood. The methods



employed have been greatly improved and now permit quantitative investigation of the androgenic content of 30 cc. of blood from human male adults. It has been found that in blood as well as in urine there is little free androgenic material. The curve of activation of androgen in blood with acid is similar to that of urine.

**A Study of the Preparation and of the Conditions for Hydrolytic Activity of Asparaginase.** BY THOMAS L. McMEEKIN. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

A highly active and stable solution of the enzyme asparaginase has been prepared by growing wild yeast (colored *Torula*) in solutions containing asparagine and one other amino acid, either alanine or glycine. As growth proceeded, additional asparagine was added until the ammonia nitrogen produced reached a concentration of about 0.39 M.

After standing 3 days, the yeasts were removed by centrifuging and filtering. The solution, containing the enzyme, was dialyzed free from ammonium salts. 1 cc. of the solution in phosphate buffer of pH 6.4 hydrolyzed 0.025 mg. of asparagine per hour at 25°, independent, over wide limits, of time and asparagine concentrations. The solution did not hydrolyze sucrose or peptides, showing the absence of invertase and peptidases.

The rate of enzymic hydrolysis of asparagine increases with time when calculated by the law applicable to a monomolecular reaction.\* The rate of hydrolysis is directly proportional to the enzyme concentration, doubles when the temperature increases from 25–35°, and increases with ionic strength up to a value of  $\mu = 0.3$ , in phosphate buffers. Ammonium aspartate, a product of the reaction, decreases the velocity in concentrations greater than 0.015 M. The velocity increases regularly with increase in pH from 5 to 9.

Asparaginase also hydrolyzes glutamine, but does not hydrolyze aliphatic acid amides. Its use in determining asparagine and glutamine in biological material, as well as the affinity of enzyme for substrate, and the influence of the dielectric constant on the course of the hydrolysis are being explored.

\* Geddes, W. F., and Hunter, A., *J. Biol. Chem.*, **77**, 197 (1928).

**Studies on the Proteins of Smooth Muscle. I.** BY JOHN W. MEHL. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

A protein has been obtained from the smooth muscle of beef intestine which shows double refraction of flow. It is extracted by salt solutions between pH 7 and 8 and with an ionic strength in the neighborhood of 1. The doubly refractive protein may be precipitated from such extracts by dialysis or dilution, by salting-out with 0.3 saturated ammonium sulfate, or by acidification of the extract. On being precipitated by any of these methods, it rapidly becomes insoluble. This change to an insoluble form is very rapid at room temperature, but is considerably retarded at low temperatures. In all these properties, as well as that of double refraction of flow, it resembles the myosin of striated muscle.

In addition to this protein, the extracts contain at least two other proteins. One of these is precipitated by one-half saturation with ammonium sulfate and has an isoelectric point of about 5.5. The other is precipitated at higher concentrations of ammonium sulfate and has an isoelectric point of about 6.4.

**Studies on the Chemical Determination of Vitamin B<sub>1</sub>.** BY DANIEL MELNICK\* AND HENRY FIELD, JR. *From the Department of Internal Medicine, University of Michigan, Ann Arbor*

Recently Prebluda and McCollum reported that vitamin B<sub>1</sub> reacts in alkaline solution with diazotized *p*-aminoacetophenone to yield a purple-red compound. We have found that xylene will quantitatively extract all this pigment and that this xylene layer lends itself to colorimetric evaluation. Furthermore, of nineteen other nitrogenous compounds adsorbable on permutit, it was observed that although some do react with the reagent to yield colored solutions, none of these is extracted by xylene. Permutit was the only adsorbent studied that will remove the vitamin and permit a 100 per cent elution with 3.5 N H<sub>2</sub>SO<sub>4</sub>, yielding a solution which can be rendered suitable for analysis. The influence of volume, varying concentrations of the reagent, temperature, pH, interfering substances, and the time required for the completion of the reaction have been investigated. With these factors controlled, the results of forty-eight determinations of the vitamin

\* Upjohn Fellow in Clinical Research.

B<sub>1</sub> concentrations (10 to 80 micrograms per cc.) have indicated excellent reproducibility; the maximal deviation from the average was  $\pm 3$  per cent. With slight modifications the reaction has been made sensitive enough to permit determination of the vitamin concentrations in solutions containing as little as 1 microgram per cc. Phenol extraction of the vitamin from saturated salt solutions has been studied. The use of this procedure coupled with the permutit adsorption and subsequent elution techniques has enabled us to test for vitamin B<sub>1</sub> in urine. However, the recoveries are unsatisfactory at present and further work is being done on this problem.

**The Isolation of a Mucoitin Disulfuric Acid from Gastric Mucin.**

BY KARL MEYER AND ELIZABETH M. SMYTH. *From the Chemistry Laboratory, Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York*

The acid polysaccharide of gastric mucin has hitherto been obtained by alkaline hydrolysis, and has been characterized as a mucoitinsulfuric acid by analysis and by the isolation of glucosamine. It is now shown that the acid fraction can be isolated without the use of strong alkali by separating it from the neutral polysaccharide as a protein salt, decomposing the protein salt with CaCl<sub>2</sub>, and isolating the acid Ca salt. From this there has been isolated a mucoitin disulfuric acid in which one of the sulfuric acid groups is very labile. The acid seems to be an isomer of heparin, but in spite of this similarity it has only about 1 per cent of the activity of pure heparin. The salts formed by this acid with proteins are much more stable than those formed by chondroitinsulfuric acid\* in accordance with the accumulation of acid groups in the molecule.

**The Semiquinone of Lactoflavin.** BY LEONOR MICHAELIS AND G. SCHWARZENBACH. *From the Laboratories of The Rockefeller Institute for Medical Research, New York*

It had been shown that lactoflavin on partial reduction yields a compound on the oxidation level of the free radical monohydro-lactoflavin. This radical is red in strongly acid solution and green

\* Meyer, K., Palmer, J. W., and Smyth, E. M., *J. Biol. Chem.*, **119**, 501 (1937).

at any pH > 2. In the meantime Kuhn and Ströbele have shown that not only one but three different compounds on oxidation levels between flavin and dihydroflavin can be obtained in the crystalline state; and Michaelis and Fetcher have shown, for a suitable dyestuff, that a semiquinone radical may be in equilibrium with its dimeric, valence-saturated compound. What is that intermediate form occurring under physiological conditions? New titration experiments with solutions of lactoflavin in varied concentrations show that in solutions of the physiologically occurring concentration range, the intermediate form is entirely represented by the free radical, whereas in higher concentrations an easily measurable amount of its dimeric form is also present. The constant of this dimerization can be measured with satisfactory accuracy. None of the other forms, described for the solid state, occurs in solution. The precision of the titration experiments is now great enough to determine the semiquinone formation constant for various pH values separately; it is no longer necessary to rely on its value as averaged over a wide range of pH in which it varies but little. Thus, an improved plot of the three normal potentials against pH has been obtained. The maximum amount of the free radical under physiological conditions turns out to be even somewhat higher than previously assumed (14 instead of 10 per cent of the total dye at pH 7, and even more at pH > 7).

**A Study of Sulfhemoglobin.** BY HARRY O. MICHEL. *From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*

The compound, sulfhemoglobin (SHb), having a characteristic absorption maximum at 620 m $\mu$ , is formed by the action of inorganic, divalent sulfur on HbO<sub>2</sub>. The sulfide sulfur is catalytically oxidized by O<sub>2</sub> in the presence of HbO<sub>2</sub>, with SHb forming at a rate equal to the rate of O<sub>2</sub> uptake. The probable mechanism of SHb formation is  $\text{HbO}_2 + \text{H}_2\text{S} = \text{Hb} + \text{S} + \text{H}_2\text{O}_2$  (1);  $\text{Hb} + \text{H}_2\text{S} + \text{H}_2\text{O}_2 \rightarrow \text{HbS} + \text{unknown products}$  (2). O<sub>2</sub> uptake measurements indicate the probable correctness of reaction (1). HbO<sub>2</sub> reduced by excess reducing reagent, as Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, does not react with S<sup>-</sup>. If now a peroxide, as H<sub>2</sub>O<sub>2</sub> or NaBO<sub>2</sub>, is added, SHb forms instantly. No other type of oxidizing reagent has been found to bring about this reaction. The catalytic effects of

$\text{Na}_2\text{S}_2\text{O}_4$ , phenylhydrazine, *p*-aminophenol, on the reaction between  $\text{HbO}_2$  and  $\text{S}^-$  to form SHb, are presumably due to their reaction with  $\text{O}_2$  to form peroxide and Hb.

Complete conversion of Hb to SHb has not been achieved. The reaction is apparently completely irreversible. Solubilities of Hb and SHb from the same species are identical in water or concentrated phosphate buffers, and their isoelectric points and cataphoretic mobilities are equal. The stability toward alkali for Hb and SHb of the same species is identical. The molecular weight is unchanged in transforming Hb to SHb (osmotic pressure measurements). SHb is a ferrous derivative and requires 1 equivalent of ferricyanide to give the ferric form. The ferric form of SHb denatures rapidly. Apparently 1 atom of S is required to produce 1 atom of SHb iron. SHb combines with CO gas to form a reversibly dissociable SHb—CO, but does not bind  $\text{O}_2$ .

**The Effect of Type and Amount of Protein on the Cataract-Producing Action of Galactose.** BY HELEN S. MITCHELL AND GLADYS M. COOK. *From the Massachusetts State College, Amherst*

The authors have previously reported that a protein deficiency (5 per cent) hastens the development of cataract in rats fed on a diet containing 25 per cent of galactose. Further work indicates that a high level of protein (45 per cent) greatly inhibits the cataractous change in the lens compared with that observed in rats fed the control ration containing 15 per cent of protein. The degree of galactemia is not significantly altered by the level of protein ingested. The striking results obtained with rations containing casein led to the use of five other sources of protein: egg albumin, lactalbumin, beef muscle, fish muscle, and soy bean meal fed at comparable levels of 5, 15, and 45 per cent. The protective action of these crude proteins measured in terms of time and incidence of mature cataract varies to a significant degree, showing egg albumin to be more protective and the others less protective than casein. With a lower level of galactose (15 per cent) the influence of the amount of protein in the diet becomes more pronounced. A high incidence of mature cataract develops when 5 per cent of protein is fed, early lens changes occur with 15 per cent of protein, and complete protection is common with 45

per cent of protein. As previously reported, the slight inhibitory effect of cystine upon the cataract-producing action of galactose does not seem to explain the protective effect of proteins. Methionine also exhibits some inhibitory action but not commensurate with that of protein containing an equivalent amount of this amino acid.

**Acid Properties of Hemin and Ferrihemic Acid.** BY DEMPSE B. MORRISON AND EDWARD F. WILLIAMS, JR. *From the Department of Chemistry, College of Medicine, University of Tennessee, Memphis*

Titration curves, solubility data, and electrophoresis experiments have demonstrated that hemin and ferrihemic acid\* (hematin) exhibit no basic groups, and are exceedingly insoluble in water or in dilute hydrochloric acid. In consideration of their molecular weights, hemin and ferrihemic acid appear to be relatively strong dibasic acids. 1 mole of hemin requires 3 moles of sodium hydroxide to form the disodium salt, while ferrihemic acid requires 2 moles; the chlorine of the hemin is titrated simultaneously with the 1st hydrogen atom and thus modifies the initial part of the titration curve as compared with that of ferrihemic acid. The absence of a break in the titration curve when the 1st hydrogen atom of ferrihemic acid or of hemin is neutralized indicates that  $K_1$  and  $K_2$  are of the same order. Addition of dilute hydrochloric acid to disodium ferrihemate solutions precipitates ferrihemic acid and not hemin. In electrophoresis of disodium ferrihemate in aqueous solution, the ferrihemate ion migrates to the anode. With a dilute alcoholic solution of hemin containing some free hydrochloric acid, the migration is much slower but in the same direction.

**Effects of Brief, Vigorous Exercise on the Electrolyte Pattern of the Blood Serum of the Dog.** BY MINERVA MORSE AND FREDERIC W. SCHLUTZ. *From the Department of Pediatrics of the University of Chicago, Chicago*

A 2 minute, vigorous run on the inclined treadmill by a trained dog produced changes in the concentrations of all of the com-

\* Use of the term "ferrihemic acid" facilitates naming of the salts, and is a more descriptive and less ambiguous name than "hematin."

ponents of the blood serum which were studied, pH, bicarbonate, lactate, phosphate, chloride, proteinate, and total fixed base.

The greatest changes occurred in bicarbonate and lactate. The fall in bicarbonate usually exceeded the rise in lactate by 2 to 4 milli-equivalents. Recovery was often delayed by as much as 6 minutes, but, after the delay, proceeded rapidly and was practically complete at the end of a half hour. 2 hours after the run, the lactate concentration was below the initial level.

Little change in phosphate was observed at the end of the run, but a marked decrease occurred in the next 10 minutes. Recovery was not complete after a half hour. After 2 hours the phosphate concentration usually exceeded the initial value.

Proteinate was usually higher at the end of the run, but fell rapidly during recovery, owing partly to pH shift, partly to decreased protein concentration.

Changes in chloride and total fixed base were small. Significant chloride changes include an increase in chloride at the end of the run whenever a rise in pH occurred, an increase in chloride during the first 10 minutes of recovery in case no pH rise occurred, and a chloride concentration about 2 milli-equivalents greater than the initial value in several instances after 2 hours recovery. Total fixed base changes usually approximated chloride changes.

**Rat Milk Fat As Affected by Lipids in the Diet. III.** BY ARTHUR J. MUELLER AND WARREN M. COX, JR. *From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana*

At parturition, ten mother rats were placed on each of ten purified diets and milked on the 18th and 20th days of lactation. The mothers were kept on the particular diets until three litters had been raised. All diets were identical save for 20 per cent of different fats, incorporated as follows: none, completely hydrogenated vegetable oil, coconut oil, butter oil, lard, olive oil, corn oil, soy bean oil, walnut oil, linseed oil.

The behavior of each of the ten mothers on each fat has been evaluated for three litters, in terms of young raised and their weight, volume of milk obtained, its percentage fat, and iodine value. The reproductive performance on 20 per cent fat is not exceptionally good. No consistent differences in favor of any one fat were observed, as measured by weight or percentage of young raised, or volume of milk secreted.

The iodine number of the milk fat followed that of the fat fed, and the relation can be expressed as a straight line function. The relationship of food fat to milk fat is shown to be as direct as that of food fat to body fat.

### **The Determination of Morphine in the Urine of Drug Addicts.**

BY FRED W. OBERST. *From the Laboratory of the United States Public Health Service Hospital, Lexington, Kentucky*

The presence of morphine in the urine of drug addicts may rapidly be determined by making two extractions with ethyl acetate and the subsequent formation of a morphine-molybdate-vanadate complex. This appears as a fine, white precipitate, remaining in suspension, the turbidity of which is roughly proportional to the morphine concentration and may be compared with a prepared standard. Morphine concentrations as low as 0.03 mg. in 25 ml. of urine can readily be detected.

Morphine may be determined quantitatively in concentrations ranging from 0.08 to 3.0 mg. per 100 ml. of urine by a colorimetric method. The morphine is extracted from the urine by the Pierce and Plant procedure, with the continuous liquid-liquid extraction process, followed by acid and alkaline extractions. The final residue is dissolved in water and further purified by means of permutit. Morphine combines with the permutit, while most other reducing substances are removed by washing. The morphine is finally liberated with sodium carbonate and its concentration determined by means of the Folin and Denis phenol reagent. A stable, blue color develops which is compared with a suitable standard.

With a liquid-liquid extractor having a capacity of 70 ml. approximately 0.4 mg. of morphine may be determined in 70 ml. of urine taken out of a 24 hour specimen from a drug addict receiving daily 200 mg. of morphine.

### **The Reticulocyte As an Index to Hematopoiesis in the Albino Rat.** BY JAMES M. ORTEN. *From the Department of Physiological Chemistry, Wayne University, College of Medicine, Detroit*

In previous studies, a remarkably uniform inverse relationship between the concentration of hemoglobin and number of erythrocytes in the blood of the rat, on the one hand, and the proportion



of reticulocytes, on the other, was observed under a number of experimental conditions. The present report deals with the predictable nature of this relationship.

When average values for the concentration of hemoglobin or the number of erythrocytes in the blood of normal growing rats are plotted against the percentage of reticulocytes, with ordinary coordinate paper, fairly regular curves are obtained. When the values are plotted on semilogarithmic paper, however, the logarithmic scale being used for the percentage of reticulocytes, approximately straight lines result.

Reticulocyte values reported by other investigators on normal growing rats correspond reasonably well with those predicted from the charts described. Further reticulocyte data on rats under different experimental conditions, including *Bartonella* anemia, and anemias incident to infections, likewise agree well with predicted values. In rats with a hypochromic type of anemia due to iron or protein deficiencies, however, deviations in reticulocyte values predicted from the erythrocyte count are observed. The usual relation of reticulocytes to the concentration of hemoglobin, on the other hand, remains unaffected. Animals with chronic polycythemia produced by cobalt frequently show reticulocyte counts below normal; these usually agree well with the predicted values.

This predictable normal relationship among reticulocytes, erythrocytes, and hemoglobin, and definite deviations therefrom, should prove useful as an index to abnormal hematopoiesis in the rat.

**The Dipeptidase of Chick Embryo Extracts.** BY A. H. PALMER AND MILTON LEVY. *From the Department of Chemistry, New York University College of Medicine, New York*

Extraction of ground 3 day chick embryos with 30 per cent glycerol yields preparations hydrolyzing one form of *dl*-alanylglycine at a pseudounimolecular rate up to approximately 80 per cent completion at 40°. The optimum pH is 7.8 and phosphates are not inhibitory. The rate constant  $k$  (time in minutes,  $\log_{10}$  used) is not proportional to the volume ( $V$ ) of extract (in constant volume of hydrolyzing mixture) but follows the relation  $k = CV^{1.86}$  where  $C$  is a characteristic of the particular extract. A

scale of dipeptidase units is constructed on this basis by defining a unit as one-third of the amount of enzyme giving  $k = 0.03$  under the conditions of the experiment.

**New Evidence That Cooking Egg White Removes a Harmful Factor Rather Than Creates a Protective Factor.** BY HELEN T. PARSONS AND DORIS JOHNSON. *From the Laboratory of Home Economics, University of Wisconsin, Madison*

A severe degree of "egg white injury" was induced in rats by feeding a diet high in raw egg white, a procedure which has been shown previously to result in a low content of protective factor in the livers and kidneys of rats. Half of these animals were then fed a ration containing 20 per cent of thoroughly steamed egg white; the other half, 66 per cent.

After a curative period of 25 days the animals were killed and the livers and kidneys of the two groups were assayed for the protective factor. The similarity of the concentrations in the organs of the two sets on unequal intakes of the steamed egg white indicated that a protective factor had not been created or released by heating the egg white.

The hypothesis stated previously, that raw egg white interferes in some way with a protective factor, whereas thoroughly cooked egg white does not, appears to be confirmed.

**A Case of Diabetes Mellitus with Remissions, Autogenous Hypoglycemia, and Tolerance for Low Blood Sugar.** BY W. D. PAUL AND R. B. GIBSON. *From the Pathological Chemistry Laboratory, University Hospital, State University of Iowa, Iowa City*

Since 1929 we have studied the case of a boy, then 15 years of age, whose hyperglycemia and glycosuria were controlled with diet and insulin when first admitted. Between April, 1929, and July, 1931, he was hospitalized five times, four in coma. Response to management after these crises was unusually good, though the tolerance fluctuated. He was given a high carbohydrate diet (July, 1931), after preliminary management as usual, to stimulate latent tolerance and then placed on a general diet without insulin. Blood sugars varied, sometimes high, most often profoundly hypoglycemic; some stabilization eventually

took place. In February, 1932, he was once more in acidotic coma. A second remission was provoked and on a general diet extreme hypoglycemia alternated with transient relapses to the diabetic status. The patient was becoming tolerant to low blood sugar. A return of the diabetes and failure to induce remission in December, 1932, was followed by reestablishment of a normal tolerance in March, 1934. This lasted 3 months. The patient then managed himself roughly with insulin. He was admitted to the hospital September to October, 1936, restored to a normal tolerance which continued 7 months and was achieved again in December to January, 1937-38.

This study supports the view, previously presented, of a latent tolerance in diabetes mellitus—a dormant rather than a functionally inadequate metabolism for carbohydrate.

### **The Solubility Precipitation Patterns of the Serum Proteins.**

BY WILLIAM A. PERLZWEIG, ALBERT A. KONDRITZER, AND ERNST BRUCH. WITH THE ASSISTANCE OF HARRIOTT I. GATES ANDERSON. *From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*

Precipitation curves of the serum proteins obtained by the use of the phosphate method of Butler and Montgomery\* are presented. The curves for normal human, horse, rabbit, dog, and rat sera show a definite constancy of pattern for each species studied, but with striking variations among the different species. In a number of pathological conditions in man in which curves were obtained a great variability of pattern was found, affecting different portions of the curve. In some, not all, cases of multiple myeloma with hyperproteinemia a sharply defined fraction was observed, corresponding to the euglobulin. Evidence is also accumulating that the pattern of the precipitation curve in a given disease is fairly constant.

From our data it is apparent that the usual methods of fractionation of the serum globulins and albumins by precipitation with few given concentrations of the neutral salts cannot yield significant or comparable values, except in normal sera of any one species.

\* Butler, A. M., and Montgomery, H., *J. Biol. Chem.*, **99**, 173 (1932-33).

**The Nature of the Physiologically Inactive Form of Androgens in Human Urine.** BY D. H. PETERSON, W. H. HOSKINS, J. R. COFFMAN, AND F. C. KOCH. *From the Department of Biochemistry of the University of Chicago and the Bauer and Black Laboratories, Chicago*

Normal butyl alcohol extracts all the androgenic material from acidified, unboiled, fresh urine of men. These extracts contain practically all the material in a conjugated, inactive form. Mild acid hydrolysis liberates the activity, but prolonged boiling with acid results in subsequent partial loss of activity. The indications are that the androgenic material consists of androsterone and dehydroandrosterone conjugated as hexuronides.

**The Rate of Carbohydrate Absorption from the Rat Intestine.**

BY H. B. PIERCE AND LORRAINE HAEGE. *From the Department of Vital Economics, The University of Rochester, Rochester, New York, and the Department of Biochemistry, College of Medicine, University of Vermont, Burlington*

Definite amounts of several cereals were fed to young adult female rats fasted for 48 hours. Animals taking more than 15 minutes to eat were discarded. Rats were killed by a blow on the head at 1, 2, and 3 hour intervals after having food placed before them. The alimentary tract was removed quickly, and the stomach and small intestine separated, slit open, and the cereal in each recovered by washing. The contents of the stomach and intestine thus obtained were analyzed for starch and free sugar.

Approximately 65 per cent of the starch fed was absorbed during a 1 hour period, 85 per cent during a 2 hour period, and 92 to 100 per cent during a 3 hour period. These data indicate that the rate of absorption decreases with time. Although measurable amounts of starch and free sugar could be recovered from the stomach after 1 and 2 hours, little of either could be recovered from the intestine at the end of the corresponding periods, indicating a rapid absorption from the intestine. The quantity of free sugar present in the stomach and intestine decreases with time.

In a series of studies in which several groups of rats of different weights were used, it was found that with cereals the total amount of carbohydrate absorbed was approximately the same for rats weighing 120 to 140 gm. as for those weighing 190 to 220 gm.

Thus, coefficients of absorption (mg. per 100 gm. of body weight per hour) were markedly different for the two groups.

The data show considerable variation in the emptying rate of the rat stomach.

**Blood Chemistry in Human Trichinosis.** BY H. B. PIERCE AND ERNEST HARTMAN. *From the Departments of Biochemistry and of Pathology and Bacteriology, College of Medicine, University of Vermont, Burlington*

This report embraces part of a study made on forty-four men hospitalized at Fort Ethan Allen subsequent to an outbreak of trichinosis at a Civilian Conservation Corps Camp at Waterbury, Vermont. The date of infestation has been established as October 27, 1937. Beginning with November 23, venous blood was obtained from these patients every 4th or 5th day for a period of 2 months. Calcium, phosphorus, cholesterol, sugar, non-protein nitrogen, and chloride were determined. Several weeks after the analyses were begun, the men were divided into two groups,—one in which each member received daily  $\frac{1}{2}$  ounce of a high grade cod liver oil plus 45 grains of calcium gluconate and another in which the men did not receive these substances. Later the amount of calcium gluconate given patients in the first group was increased to 90 grains *per diem*.

The calcium to phosphorus ratios were found to be low early in the experiment, owing primarily to a high phosphorus and slightly low calcium. These ratios tended to become higher in both groups as time progressed. The values for cholesterol show little of significance. Sodium chloride was below normal in some of the patients, particularly during the early part of the investigation. Blood sugars averaged low normals, whereas non-protein nitrogen approached the higher normal limits.

**The Concentrations of Sodium, Potassium, and Chloride in Plasma and Urine during Short Periods of Low Sodium and Elevated Potassium Intake.** BY MARSCHELLE H. POWER, RUSSELL M. WILDER, AND HAYDEN H. CUTLER. *From the Department of Biochemistry and the Department of Medicine, The Mayo Foundation, Rochester, Minnesota*

Thirty-six individuals were given a diet containing about 0.60

gm. of sodium and 4.0 gm. of potassium per day. On the 1st day additional potassium, 0.033 gm. per kilo of body weight, was given during the afternoon. On the 2nd day a sample of venous blood was taken before breakfast, additional potassium was given throughout the morning, and the fluid intake was increased to the equivalent of 40 cc. of water per kilo. On the 3rd day a sample of blood was taken at 10.00 a.m., and fluid equivalent to 20 cc. of water per kilo was given before 11.00 a.m. The urine was collected during the 2nd day and up to 12 noon on the 3rd day.

The average concentrations of sodium, potassium, and chloride in the plasma of twenty-eight persons who did not have adrenal insufficiency were 136, 4.6, and 98.5 milli-equivalents per liter, respectively, for the first sample of blood, and 134, 4.7, and 95.6 milli-equivalents for the second sample of blood. In eight cases of adrenal insufficiency (Addison's disease) these concentrations were 130, 5.1, and 94.8 milli-equivalents in the first sample of blood, and 122, 5.5, and 88.6 milli-equivalents in the second sample of blood. When the concentrations of sodium and chloride in the plasma had descended to these low levels, more or less characteristic signs of adrenal insufficiency had developed in many of these cases.

The average urinary excretion of sodium and chloride in the series of cases of adrenal insufficiency was considerably greater than that in the control group.

**The Oxidation-Reduction Potentials of the Oxidation Products of Inositol.** BY PAUL W. PREISLER, EDGAR S. HILL, ETHEL RONZONI, AND LESLIE YOUNG. *From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis*

When the cyclic compound inositol,  $(\text{CHOH})_6$ , present in many plant and animal tissues, is oxidized by suitable means, 12 hydrogen atoms are removed, forming triquinoyl,  $(\text{CO})_6$ , which can be reduced by adding 2 hydrogen atoms for each step to rhodizonic acid,  $(\text{CO})_4(\text{COH})_2$ ; tetrahydroxyquinone,  $(\text{CO})_2(\text{COH})_4$ ; and hexahydroxybenzene,  $(\text{COH})_6$ . The series possesses the unusual feature of having three reversible oxidation-reduction steps, with 6 equivalents total, of the quinone-hydroquinone type on the same molecule, whose relationships

present a new phase in oxidation-reduction potential theory. On reducing rhodizonic acid, two separated curves, each with index potential ( $E_2 - E_1$ ) of 0.014, were obtained, indicating very little semiquinone formation; in more acid buffers the two curves progressively merge into one with index potential of 0.018 volt at pH 5 for the compound curve. At pH 9.88 the  $E'_0$  values are  $-0.463$  for rhodizonic acid-tetrahydroxyquinone and  $-0.274$  volt for tetrahydroxyquinone-hexahydroxybenzene, being  $+0.320$  and  $-0.131$  volt to the hydrogen electrode at this pH. The third step, triquinoylrhodizonic acid, presents some difficulties because of the insolubility and instability of the components.

At higher pH than 11, rhodizonic acid is converted by the alkali into a 5-carbon ring compound, croconic acid hydride, which on oxidation yields croconic acid,  $(\text{CO})_3(\text{COH})_2$ ; further oxidation forms leuconic acid,  $(\text{CO})_6$ . Croconic acid reduces to give hydrocroconic acid  $(\text{CO})(\text{COH})_4$ . These compounds and their 6-carbon ring relatives resemble certain sugar derivatives in being cyclic and having  $:\text{CO}$  and  $\cdot\text{COH}$  groups and certain of their  $E'_0$  are of the order of potential given by sugar solutions. The oxidation-reduction potentials of these 5- and 6-carbon ring systems are being measured at various pH values.

## The Estimation of Albumin and Globulin in Blood Serum. II.

**Separation of Fractions by Centrifugation with the Angle Centrifuge.** BY J. WAIDE PRICE, HOWARD W. ROBINSON, AND CORINNE G. HOGDEN. *From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati*

In a previous communication we have shown that in the separation of the globulin precipitate from the concentrated sodium sulfate solution by filtration through filter paper a definite amount of the soluble protein was adsorbed by the paper. In order to avoid errors from this source it was found necessary to discard the first portions filtered.

We have found that with the angle centrifuge, introduced by Lundgren of Sweden, filtration through filter paper can be avoided in most cases. The protein-sodium sulfate mixtures are placed in the flat, elliptical, 15 cc. heavy Pyrex tubes, covered with a rubber cap, and centrifuged at a speed of 4200 to 4500 R.P.M.

The precipitated particles settle rapidly, as they travel only a short distance before reaching the outside wall of the tube. Analyses of the supernatant fluid, obtained by centrifugation, give values that agree, within experimental error, with the "correct values" obtained with our recently described filtration procedure. Half an hour's centrifuging seems to be sufficient for most mixtures, although 1 to 2 hours gives a firmer packing of the precipitate.

**The Vitamin A and D Content of Halibut and Sable-Fish Visceral Oil.** BY L. I. PUGSLEY. *From Pacific Fisheries Experimental Station, Fisheries Research Board of Canada, Prince Rupert, Canada*

The oil obtained from the viscera (exclusive of the liver and stomach) of the Pacific halibut (*Hippoglossus hippoglossus*) and the sable-fish (*Anaplopoma fimbria*) has been found to be relatively high in vitamin A as compared to the liver oil. The vitamin A content as measured by the  $\text{SbCl}_3$  reaction varied between 40,000 and 450,000 blue units per gm., whereas the corresponding liver oil varied between 15,000 and 50,000 blue units per gm. The yield of oil (by ether extraction) from the stomach was low (0.1 per cent), whereas the yield from the pyloric ceca or intestine varied from 4 to 8 per cent. The viscera, exclusive of the stomach and liver, constitute 3 to 4 per cent of the fish.

Vitamin D assays have been carried out on three lots of halibut visceral oil and the values were found to vary between 200 and 500 international units. One sample of sable-fish visceral oil assayed 200 international units per gm. These values are relatively lower than usually obtained for the liver oils of these fish.

**Acrodynia and the Essential Fatty Acids.** BY F. W. QUACKENBUSH AND H. STEENBOCK. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

On a basal diet of purified casein, glucose, and salts, supplemented with carotene, calciferol, synthetic vitamin  $\text{B}_1$ , and lactoflavin, rats developed an acute dermatitis and a condition which has been described by others as acrodynia. In acute cases the mouth, fore paws, and eyes were involved especially. In chronic cases



the paws often became gangrenous, the ears became swollen and encrusted, and minute scales appeared in abundance over the entire body. In experiments involving over 500 rats, no animals failed to develop the symptoms within 4 to 5 weeks.

Complete healing of either the acute or chronic symptoms was obtained by daily administration of 10 mg. of wheat germ oil, corn oil, or Wesson oil, 200 mg. of coconut oil, or 500 mg. of butter fat. Cures were not effected by the addition of 10 per cent of hydrogenated coconut oil to the diet.

The unsaponifiable fraction from wheat germ oil was devoid of potency, the ethyl esters prepared from the soap fraction containing the entire activity. When the unsaturated fatty acids were fractionally crystallized from acetone; the highest activity was obtained with the fraction separating between  $-60^{\circ}$  and  $-75^{\circ}$ . 10 mg. of ethyl linolate (Rollett) per day gave complete healing.

Cured animals maintained themselves free of all symptoms as long as the fat supplement was continued or until the end of the survival period, which was usually several months.

**The Effects of Acids upon Carotenoids.** BY F. W. QUACKENBUSH, H. STEENBOCK, AND W. H. PETERSON. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

Five new carotenoids, designated for convenience as Pigments A, B, C, E, and F, were isolated from alfalfa silage and from acidified fresh alfalfa. While they were not detected in the alcoholic extracts from fresh green alfalfa, their presence was demonstrated after the extracts had been treated with acids.

A quantitative separation of the pigments was effected chromatographically with MgO and benzine-alcohol mixtures. A partial separation of the pigments was also obtained by their differential phasic distribution between benzine and 85 per cent EtOH. Pigments A, B, and C were predominantly epiphasic; Pigments E and F were hypophasic. The absorption curves of Pigments A, B, and E were essentially identical with those of lutein; those of Pigments C and F failed to show well defined maxima.

Pigments A, B, and E were produced from lutein, in large amounts by  $N/40$  HCl and  $H_2SO_4$ , and in smaller amounts by

N/40 lactic or oxalic acid. The stronger acids favored the production of Pigment B; weaker acids the production of Pigment A.

Pigments A and B exhibited no biological activity when fed to vitamin A-deficient rats. Both pigments were found in butter fat produced by cows on A.I.V. silage.

Inasmuch as the usual methods of carotene analysis fail to differentiate between carotene and Pigments A, B, and C, the values obtained by their use on silages, especially those prepared with mineral acids, are obviously too high.

**Qualitative Differences in the Prothrombin, Thromboplastin, and Thrombin of Different Species.** BY ARMAND J. QUICK.  
*From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

There is no demonstrable qualitative difference in the prothrombin of man, rabbit, cat, dog, horse, or monkey; all are activated by thromboplastin obtained from any species of this group. The prothrombin of the guinea pig is readily activated by its own thromboplastin, but the latter acts slowly on rabbit or human prothrombin in converting it to thrombin. Thromboplastin prepared from the brain of the goose will rapidly convert goose prothrombin to thrombin, but it has only a feeble action on rabbit or human prothrombin; and *vice versa*, rabbit thromboplastin has a weak activating power on the prothrombin of the goose and the chicken. Thrombin prepared from the blood of various warm blooded animals readily clots fibrinogen from bloods of the same group without any evidence of species specificity. The prothrombin and thromboplastin of turtle blood show a marked but not an absolute difference from that of mammalian blood, and turtle thrombin acts more rapidly on its own fibrinogen than on that obtained from warm blooded animals.

**Therapeutic Effect of Diaminodiphenyl Sulfide, Diaminodiphenyl Sulfone, and Related Compounds in the Treatment of Mice Infected with  $\beta$ -Streptococcus haemolyticus.** BY GEORGE W. RAIZISS, M. SEVERAC, J. C. MOETECH, AND LEROY W. CLEMENCE. *From the Graduate School of Medicine, University of Pennsylvania, and the Dermatological Research Laboratories, Philadelphia*  
Buttle, Stephenson, Smith and Foster, and Fournau, Trefouel,

Nitti, and Bovet reported almost at the same time that diaminodiphenyl sulfide and diaminodiphenyl sulfone have greater therapeutic effect in the treatment of mice infected with  *$\beta$ -Streptococcus hæmolyticus*. We have studied the toxicity and therapeutic effects of the chemical compounds mentioned above, and have found that their therapeutic effects were greater than that of sulfanilamide.

Mice were infected intraperitoneally with about 1000 minimum lethal doses of a virulent culture of  *$\beta$ -Streptococcus hæmolyticus* (Strain C-203) which killed mice in a dilution  $10^{-8}$  (1:100,000,000). Controls used in every experiment showed a death rate of 100 per cent. The drugs were given  $1\frac{1}{2}$  hours later *per os*. Similar treatment was continued daily for 4 additional days. The mice were kept under observation for about a month.

The toxicity of diaminodiphenyl sulfide and diaminodiphenyl sulfone was found to be greater than that of sulfanilamide when given to rabbits *per os*. The diacetyldiaminodiphenyl sulfide was found to be less toxic than sulfanilamide. The diacetyldiaminodiphenyl sulfone was found to be the least toxic of all. The diaminodiphenyl sulfide and particularly the diaminodiphenyl sulfone exerted a greater therapeutic effect than sulfanilamide. The diacetyl compounds also seemed to be more therapeutic than sulfanilamide. In view of the higher therapeutic effect, the diaminodiphenyl sulfide and the diaminodiphenyl sulfone, their diacetyl derivatives, and some new derivatives which we have prepared deserve further laboratory and clinical investigation.

#### **Chemical Topography of the Brain.** BY LOWELL O. RANDALL.

*From the Research Service of the Worcester State Hospital, Worcester*

In an attempt to relate functions of the brain to the chemical composition, a systematic chemical analysis was made of twenty-three brains from normal and psychotic subjects. The mean water content was higher in the gray areas—the frontal cortex, parietal cortex, and, caudate nucleus—than in the white areas—the corona radiata, frontal white, and parietal white—while the brain stem and thalamus had intermediate values. Total lipid, acetone-soluble lipid, total cholesterol, free cholesterol, phospholipid, phospholipid fatty acid, lipid phosphorus, and lipid nitrogen were higher in the white areas than in the gray areas and

intermediate in the mixed areas. The iodine number of the phospholipid fatty acids was higher in gray than in white tissues. The acid-soluble nitrogen, creatine, inorganic phosphorus, protein nitrogen, and total nitrogen were higher in gray than in white tissue and were intermediate in the mixed tissues. Only acid-soluble phosphorus and ester phosphorus had a similar distribution over all the areas. No differentiation could be made in any of the constituents between various gray areas nor between the various white areas. The higher content of protein, acid-soluble constituents, and water and the greater proportion of highly unsaturated fatty acids in gray than in white tissue signify the greater importance of these compounds in cellular activity. The higher lipid content of the white tissues signifies the greater importance of these compounds for the conducting systems of the brain.

**The Effect of Phospholipid Ingestion upon the Respiratory Quotient in Man.** BY RAYMOND REISER AND FREDERIC M. HANES.  
*From the Department of Medicine, Duke University School of Medicine, Durham, North Carolina*

The respiratory quotient, urinary inorganic phosphate, and total nitrogen, blood sugar, serum inorganic phosphate, and phospholipid were determined after the ingestion of 60 gm. of mixed soy bean phosphatides and after equivalent amounts of olive oil, disodium glycerophosphate, and a mixture of olive oil and disodium glycerophosphate. In all cases there was a lowering of the R.Q. during the first half hour after feeding. After the ingestion of phospholipid, of olive oil plus disodium glycerophosphate, and sometimes after olive oil alone, the R.Q. then quickly rose above the basal level, reaching its maximum value in 3 to 5 hours. After the ingestion of disodium glycerophosphate the R.Q. remained below the basal level.

On several occasions after phospholipid feeding the R.Q. rose to, or close to, unity. This effect on the R.Q. was not so marked after oil or oil plus phosphate, but was slightly greater in the latter. No correlation could be found between R.Q. changes and blood chemical changes. There was no significant change in blood sugar. A pronounced lowering of urinary inorganic phosphate was noted after feeding olive oil, reaching a minimum value 1 hour after ingestion.

These data may be explained by an early partial oxidation of a

part of the fat undergoing absorption, occurring possibly in the intestinal mucosa, the oxidation being completed later elsewhere in the body. The formation of phospholipid would appear to be an intermediate step in the preliminary partial oxidation.

**Biological Studies on Amino Acids with the Aid of Deuterium As an Indicator.** BY D. RITTENBERG, G. L. FOSTER, AND RUDOLF SCHOENHEIMER. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

The body fluids of two groups of mice were kept at a deuterium content of 1.5 atom per cent for 10 and for 98 days. From the carcasses ten and nine individual amino acids were isolated respectively. All of them with the exception of lysine contained deuterium. In the animal body deuterium can enter amino acids in positions from which it was not removed by protracted treatment with boiling aqueous mineral acids. No evidence was found that the deuterium in these amino acids arises from enzymatic exchange reactions in the animal. On repeating the experiments of Stekol and Hammill on the uptake of deuterium into tyrosine during tryptic digestion of casein in heavy water, we found that the tyrosine isolated contained the same minute amounts of deuterium as was found in tyrosine which had merely been dissolved in  $D_2O$ . Experiments on the biological formation of hippuric acid have shown that by using appropriate methods even glycine, which has no "completely stable" deuterium, can be followed in the animal organism. After administration of deuteroglycine and benzoic acid to rats, deuterohippuric acid was isolated from the urine.

**The Effect of Ammonolyzed Foods on the Growth of Albino Rats.**

BY RICHARD G. ROBERTS AND HERMAN J. HORVITZ. *From the Department of Physiological Chemistry, Chicago Medical School, Chicago*

That urea and ammonium carbonate may be effectively used as dietary nitrogen supplements in ruminant animals has been reported by various workers. We have previously shown that proteins are ammonolyzed in liquid ammonia, and thereby increase their nitrogen content. The present investigation was

undertaken to determine whether ammonolyzed proteins and other foods can be used as a source of nitrogen in the diet of rats. Preliminary experiments showed that ammonolyzed casein caused a loss of weight in young rats when it was used to replace other proteins. In order to determine the cause of this toxic effect, a series of experimental feedings was made in which different constituents of the diet were treated with liquid ammonia or ammonium hydroxide or all of the constituents of the diet were mixed with ammonium carbonate. It was found that failure to grow occurred in all cases in which the vitamin B complex in the form of dry yeast was allowed to come in contact with ammonia. However, if the yeast was supplied in a separate container and the remaining food ammonolyzed, the rats not only showed no such interference with growth, but actually grew more rapidly than control animals on normal diets. The results indicate that the deleterious effect of liquid ammonia or ammonia gas is due to the action of the ammonia upon the vitamin B complex. In addition it was found that ammonolyzed food has less tendency to spoil or become mouldy than untreated foods.

**The Determination of Vitamin C As Furfural through the 2,4-Dinitrophenylhydrazine Derivative of Dehydroascorbic Acid, with Studies by This Method.** BY JOSEPH H. ROE. *From the Department of Biochemistry, School of Medicine, George Washington University, Washington*

Norit filtrate of acid-extracted tissue is treated with saturated 2,4-dinitrophenylhydrazine in  $N$  HCl. The resulting osazone is heated with 12 per cent HCl containing 10 per cent  $SnCl_2$  in an autoclave at 15 pounds pressure for 30 minutes. This treatment reduces the yellow nitro derivative to a colorless compound, hydrolyzes the dehydroascorbic acid-hydrazine linkage, and converts the dehydroascorbic acid into furfural. The latter is determined by the aniline acetate technique previously described. This method appears to be entirely specific for vitamin C. The vitamin C values of plant tissues determined by this procedure thus far are in close agreement with those obtained by indophenol titration. With some animal tissues and urine the indophenol titration values are considerably higher than those obtained by this method.

**The Enzymatic Hydrolysis of Wool.** BY JOSEPH I. ROUTH.

*From the Laboratory of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*

Natural, long fiber wool was attacked very slowly by commercial trypsin and finely chopped fibers were not digested more rapidly. Powdered wool, produced by prolonged grinding of wool in a ball mill, was attacked at a greatly increased rate. In this finely ground condition, wool was appreciably soluble in water and the dissolved nitrogen and sulfur were present in the same ratio as in the natural wool. As judged by the formol titration, trypsin was effective in the hydrolysis of powdered wool, and, as judged by increases in the non-precipitable nitrogen fraction, pepsin was also effective. Powdered wool was digested approximately one-half as fast as was casein under comparable conditions.

Kerateines, produced by the reduction of wool with alkaline thioglycolate solutions, were likewise digested by trypsin and pepsin. Their rates of digestion by these enzymes did not differ significantly from that of casein under the same conditions.

**The Effect of Certain Oils in Alleviating Localized Erythematous Dermatitis (Acrodynia or Vitamin B<sub>6</sub> Deficiency) in Rats.** BY

W. D. SALMON. *From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn*

Rats receiving a fat-free diet supplemented with carotene, vitamin D concentrate, thiamine, riboflavin, and a limited amount of aqueous extract from brewers' yeast, which has been subjected to dry heat treatment (24 hours at 120-130°) before extraction, develop a severe form of erythematous dermatitis. The further addition of corn oil, linseed oil, wheat germ oil, or the fatty acids of linseed or soy bean oil cures or prevents the dermatitis. If the heated yeast extract is omitted, however, the oils do not cure or prevent the condition. Moreover, the use of corn-starch as the source of carbohydrate in the diet instead of sucrose does not prevent the onset of the dermatitis unless the heated yeast extract is likewise included.

It appears that the effective oils do not contain the entire dermatitis-preventing factor but may contain an essential part of the factor which supplements the heated yeast extract.

The methyl esters of linoleic or linolenic acid are less effective

than the oils or their total fatty acids in curative tests but do delay the onset of symptoms in preventive tests. Cod liver oil or coconut oil has relatively little activity. Nicotinic acid alone or in combination with the other supplements has no effect on the course of the disease.

### **Differences in Response to Vitamin D<sub>2</sub> of Rats on Cereal Low Phosphorus Diets and Synthetic Low Phosphorus Diets.**

BY H. SCHNEIDER AND H. STEENBOCK. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

It is now realized that cereal rachitogenic diets, although satisfactory for the production of rickets in rats, are not satisfactory for studies of phosphorus metabolism, especially as it is influenced by vitamin D. This is due to differences in availability of the phosphorus compounds present and has led to the preparation of a diet offering more positive control over the amounts of phosphorus absorbed.

A diet of purified foodstuffs low in phosphorus and free from vitamin D but complete in other dietary essentials has been devised ( $P = 0.04$  per cent,  $Ca:P = 14.3$ ). The adequacy of the nutritional components of this diet, other than P and vitamin D, has been demonstrated by successfully supporting rats for two generations after the addition of adequate phosphorus and calciferol ( $P = 0.41$  per cent,  $Ca:P = 1.4$ ).

Growth experiments have demonstrated increased rachitogenic properties of the diet over those of cereal types. However, growth was arrested when vitamin D<sub>2</sub> was added. Tissue analyses and Ca and P balances showed that this halt of growth was due to a preferential retention of P by bone and blood which limited the growth of the soft tissues.

This preferential action of vitamin D<sub>2</sub>, so pronounced that growth of the soft tissues was halted, could not be demonstrated on cereal rachitogenic diets, apparently because in the latter the quantity of phosphorus rendered available was great enough to fulfil demands of bone and blood and still allow growth of soft tissue.

### **Exploratory Experiments on the Application of the Nitrogen Isotope N<sup>15</sup> to the Study of Intermediary Metabolism. By**



RUDOLF SCHOENHEIMER, G. L. FOSTER, D. RITTENBERG, AND S. RATNER. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

The use of the nitrogen isotope requires methods of organic synthesis economical with respect to the valuable isotope. Methods of Knoop and Gabriel for amino acid synthesis have been modified to conform with this requirement. By catalytic reduction of the appropriate  $\alpha$ -keto acids in the presence of ammonia, alanine, phenylalanine, glutamic acid, aspartic acid, tyrosine, and norleucine have been prepared, all containing 2.34 atom per cent  $N^{15}$ . Glycine was prepared by the Gabriel synthesis. Analysis of the isotopic ratio  $N^{14}/N^{15}$  was made on gaseous nitrogen with a mass spectrometer.

Two general types of biological experiments have been carried out. Hippuric acid formation was followed by administering to animals glycine containing both  $N^{15}$  and deuterium. In two other experiments the utilization by rats of administered ammonia for the biological preparation of amino and guanido groups (arginine and creatine) was investigated.

**The Influence of Environmental Temperature on the Body Temperature-Lowering Properties of 1,5-Diphenylpyrazoline-3-Carboxylic Acid.** BY FRED SCHULTZ AND ROBERT M. HILL. *From the Laboratory of Biochemistry, University of Colorado School of Medicine, Denver*

The sodium salt of 1,5-diphenylpyrazoline-3-carboxylic acid lowers the body temperature of normal albino rats. We have observed that the effect is greater in winter than when the experiment is carried out in hot summer weather. To test whether this is an effect of environmental temperature, experiments were carried out at 41.1–42.2°, at 23.8–26.1°, and at 6.6°. The salt was administered in 1.5 per cent solution intraperitoneally in doses of 225 mg. per kilo of body weight. Sixteen animals were used at each temperature, eight receiving the drug and eight receiving an equivalent volume of normal saline. The animals were fasted 24 hours; the rectal temperatures were taken; and the animals were then placed at the experimental temperature about 1 hour before the drug was administered. Temperatures

were taken again just before the drug was given and at hourly intervals thereafter until the end of the experiment. In the hot room there was no significant difference in the average temperature change in the experimental and control animals. At 23.8–26.1°, though there was no change in the body temperature of the control animals, the temperature of the experimental animals fell on the average 3.0°. In the cold room, there was no significant change in the temperature of the controls. The eight experimental animals suffered drops in body temperature which averaged 19.5°. Six of these animals died. The other two which were removed to a warm room at 38.3° recovered rapidly and lived normally until they were sacrificed for autopsy 6 days later.

**Comparison and Assay of Estradiol, Estrone, and Estriol.** BY  
ELMER L. SEVRINGHAUS, CARL G. HELLER, HENRY LAUSON,  
AND JUNE B. GOLDEN. *From the Department of Medicine,  
University of Wisconsin Medical School, Madison*

The quantitative increase in weight of 320 uteri of 21 day-old rats with increasing doses of estradiol, estrone, and estriol was investigated. 0.5 cc. doses of water solutions of the estrogens were administered subcutaneously twice daily for 3 days and the rats killed 72 hours after the first injection. Body, uterine, vaginal, and ovarian weights and vaginal introitus were determined.

Minimal uterine weight increases (two of four animals responding above maximal control weight range) occurred at 0.025, 0.55, and 0.125 micrograms for estradiol, estrone, and estriol respectively, while minimal vaginal opening (one-third of the animals responding) did not occur for the same estrogens until 0.20, 4.0, and 0.5 microgram levels were reached.

Fourteen rats on each of eight dose levels of estradiol gave the following uterine weight responses. • At 0.025 microgram the uteri weighed 27.5 mg.; at 0.05, 36.1 mg.; 0.1, 50.0 mg.; 0.15, 62.3 mg.; 0.2, 67.4 mg.; 0.3, 88.1 mg.; 0.4, 92.8 mg.; and 0.75, 88 mg. For estrone fifteen rats on each of seven dose levels responded as follows: 1.0 microgram, 40.3 mg.; 2, 52.7 mg.; 3, 60.2 mg.; 4, 77.6 mg.; 6, 91.6 mg.; 8, 101.8 mg.; 10, 105.4 mg. The control weight is 20 mg. Although estradiol is about 20 times as efficient as estrone in eliciting a uterine response, corresponding increments of

dose produce corresponding increments of weight. Thus, except at the top dose levels, the two curves are similar. The response to estriol, however, reaches a maximum at approximately 50 mg. at the 1.0 microgram dose level. Further increase in dosage up to 16 micrograms does not increase uterine weight beyond 50 mg.

**Carcinogenic Activity of Some Anthracene Derivatives.** By

M. J. SHEAR. *From the Office of Cancer Investigations, United States Public Health Service, Harvard Medical School, Boston*

In further collaborative studies (Fieser and coworkers; Newman and coworkers) on the carcinogenic activity of polycyclic compounds, the effect of a number of anthracene derivatives was investigated in pure strain mice.

The rapid production of sarcomas by crystalline 10-methyl-1,2-benzanthracene, reported in a previous communication, was confirmed upon repetition. Similar results were also obtained with a dilute solution in lard. In agreement with the findings in London, the development of skin tumors was comparatively slow.

The importance of the 10-position was further established by the action of 10-ethyl-1,2-benzanthracene which was strongly carcinogenic, although the latent period was longer than with 10-methyl-1,2-benzanthracene. Tumors were also obtained with 10-methoxy-1,2-benzanthracene.

7-Methyl-1,2-benzanthracene produced only one tumor (nineteen mice) in 14 months, whereas 9-methyl-1,2-benzanthracene produced seventeen tumors (twenty mice) in 8 months, and 5,9-dimethyl-1,2-benzanthracene produced eighteen tumors (twenty mice) in 4 months. The latter acted about as rapidly as methylcholanthrene.

Reducing the dose of 4,10-ace-1,2-benzanthracene from 10 to 0.1 mg. resulted in diminished ulceration and in increased tumor production. 1',2',3',4'-Tetrahydro-4,10-ace-1,2-benzanthracene also produced tumors.

Ethylcholanthrene is strongly carcinogenic but the latent period is longer than with cholanthrene or methylcholanthrene.

In mice treated with 2-aminoanthracene to ascertain whether bladder tumors would be induced, changes were noted in the liver, including liver cell tumors.

Pellets of methylcholanthrene were implanted into the internal

organs of mice (in collaboration with A. M. Seligman). Tumors of the brain and of the spleen have been obtained, with the pellets intact in the interiors of the tumors induced.

No tumors were obtained with triphenylbenzene even after 20 months.

**An Investigation of the Nature of the Hypophyseal Ketogenic Principle.** BY R. A. SHIPLEY.\* *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The present study indicates that the ketogenic principle of the anterior pituitary is not present in appreciable quantity in protein-free ultrafiltrates passing through 8 per cent acetic acid-collodion. Treatment of crude alkaline pituitary extract with 4 volumes of acetone at pH 3.5 yields a precipitate containing the thyrotropic and gonadotropic hormones. The growth, lactogenic, ketogenic, and possibly adrenotropic principles are left in solution and may be precipitated by increasing the acetone concentration to 90 per cent. The latter fraction, when brought to pH 6.8, yields a precipitate which is extremely potent in growth activity and which contains only a trace of lactogenic principle, while another fraction which precipitates at pH 5.5 has strong lactogenic activity and is quite weak in growth-promoting power. Both of these fractions stimulate ketone production in the fasting rat but they are not as potent as the original crude extract in terms of protein concentration. Adrenotropic and lactogenic preparations made by the Lyons acid acetone extraction are entirely devoid of activity. The conclusion, therefore, seems fair that the ketogenic principle is not identical with any of the other known hormones.

The various fractions above have been tested for their effect on carbohydrate metabolism as manifested by the production of glycosuria in the partially depancreatized rat. Ketogenic and glycosuric activity run parallel in occurrence which suggests that the two effects may depend on the presence of an identical substance or two substances chemically similar.

**Production of a Deficiency in Sulfur-Containing Amino Acids by the Administration of Iodoacetic Acid.** BY ELIZABETH E.

\* National Research Council Fellow in Medicine.

SIMON AND ABRAHAM WHITE. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The inhibitory effect of iodoacetic acid on certain body processes has often been attributed to the combination of iodoacetic acid with a sulfhydryl compound which may be essential for these processes. These conclusions have been based, for the most part, on *in vitro* investigations. It has been the object of the present studies, therefore, to attempt to demonstrate *in vivo* a combination of iodoacetic acid with sulfur-containing substances which are of biological importance. It has been possible to restrict markedly the growth of young rats, ingesting a relatively low protein diet, by incorporating 1 part of iodoacetic acid into each 1000 parts of the basal diet. Under these dietary conditions, the animals cease growing. The superimposition of *l*-cystine, *dl*-methionine, or *l*-cysteine hydrochloride on the basal diet already containing the iodoacetic acid results in a prompt resumption of growth. On the other hand, the administration of taurine, inorganic sulfate, or phenyluraminocystine does not stimulate growth under the experimental conditions employed. Experiments with other types of supplements are being conducted. Studies of the effect of iodoacetic acid on the distribution of urinary sulfur are also in progress in an attempt to obtain conclusive evidence that iodoacetic acid, *in vivo*, effects a depletion of the organism's reserves of sulfur-containing amino acids by combination with sulfhydryl.

**Thermodynamic Properties of Some Amino Acids and Peptides in Aqueous Solution.** BY ELIZABETH R. B. SMITH. *From the Laboratory of Physiology, Yale University School of Medicine, New Haven*

The activity coefficients of series of amino acids and of peptides have been determined, at 25°, by an isopiestic method previously described. With an increase in the distance between the  $\text{NH}_3^+$  and  $\text{COO}^-$  groups, *i.e.* in the dipole moment, the activity coefficients decrease for both series of compounds studied,  $\alpha$ - and  $\beta$ -alanine, and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -aminobutyric acids. With an increase in the length of the carbon chain the activity coefficient increases. The fact that the activity coefficients of glycine, of  $\gamma$ -aminobutyric

acid, and of  $\epsilon$ -aminocaproic acid are similar at low concentrations is consistent with these two apparent trends.

In the peptides the effects of increases in carbon chain and in dipole moment, as well as of the characteristic linkage, are all observed; the activity coefficients of glycylglycine are markedly lower than those of glycine, and the values for triglycine are appreciably lower than those for glycylglycine. However, the peptides retain the distinctive effects of the constituent amino acids, for the activities of alanylglycine solutions are distinctly higher than those of glycylglycine solutions, as might be expected from the differences shown between glycine and alanine.

These results indicate that dipole moment, length of carbon chain, and presence or absence of other polar groups are all factors in determining the activity coefficients of amino acids and peptides.

**The Aerobic Utilization of Pyruvic Acid by Bakers' Yeast.** By C. V. SMYTHE. *From the Laboratories of The Rockefeller Institute for Medical Research, New York*

Bakers' yeast cells utilize pyruvic acid from 2 to 4 times as rapidly under aerobic conditions as under anaerobic conditions. The aerobic utilization produces large amounts of  $\text{CO}_2$  and uses comparatively small amounts of  $\text{O}_2$ ; *e.g.*, during a 1 hour experiment 612 c.mm. of pyruvic acid were utilized, 1320 c.mm. of  $\text{CO}_2$  were produced, and 565 c.mm. of  $\text{O}_2$  were absorbed. A control without added substrate, brought to the same pH with HCl, used 216 c.mm. of  $\text{O}_2$  and produced 205 c.mm. of  $\text{CO}_2$ . The  $\text{O}_2$  used in the presence of the pyruvic acid is not sufficient to produce the amount of  $\text{CO}_2$  obtained. A part of the  $\text{CO}_2$  must have been produced at the expense of some other oxidant; *i.e.*, some reduction product must have been formed. Examination of the solution in which the cells were suspended always revealed the presence of some acetaldehyde (126 c.mm. in the above experiment), but no reduction product. Examination of the cells, after thorough washing with water, showed (1) that the total carbohydrate in the pyruvic acid cells is never greater, and in 1 hour experiments is usually less, than in the HCl control (both decrease during the experiment) and (2) the amount of ether-extractable material is increased in the pyruvic acid cells. This indicates that the reduction product formed is fat.

**The Influence of Riboflavin and Certain Synthetic Flavins on the Growth of Lactic Acid Bacteria.** BY E. E. SNELL AND F. M. STRONG. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

Eight out of eleven species of lactic acid bacteria proved capable of growing on repeated subculture in a peptone medium rendered free of riboflavin by prolonged photolysis under strongly alkaline conditions. Another growth substance destroyed by this treatment and needed by all the species tested was supplied by adding small amounts of a flavin-free concentrate from liver. Since growth of the organisms paralleled their fermentative activity, the effectiveness of various media in allowing growth was measured by titrating the acid produced. Among the organisms not requiring riboflavin for growth on the above medium were *Streptococcus lactis*, *Lactobacillus arabinosus*, *Lactobacillus pentosus*, and *Leuconostoc mesenterioides*. *Lactobacillus delbrückii*, *Lactobacillus casei*, and *Bacillus lactis acidii* grew only when riboflavin was added to the medium. The latter two could be subcultured indefinitely in the medium containing riboflavin.

The indispensability of riboflavin for *L. casei* and *B. lactis acidii* offered an opportunity for verifying the reported biological activity of various synthetic flavins. Addition of lumichrome, lumiflavin, 9-*l*-arabitylisoalloxazine, 6,7-dimethylsorbitoflavin, 6,7-dimethyl-*l*-araboflavin, 6,7-dimethyl-*d*-araboflavin, 6-ethyl-7-methyl-*l*-araboflavin, or 5,6-benzo-*d*-riboflavin to the medium failed to allow growth. Addition of 6-ethyl-7-methyl-*d*-riboflavin, 6-methyl-*d*-riboflavin, or 7-methyl-*d*-riboflavin allowed growth of both *L. casei* and *B. lactis acidii*, which continued on repeated subculture, but was in no case as heavy as with natural riboflavin.

6,7-Dimethyl-*l*-araboflavin, 6,7-dimethyl-*d*-araboflavin, and 5,6-benzo-*d*-riboflavin gave small but definite increments in the titratable acidity when added to a medium containing suboptimal amounts of riboflavin. These flavins therefore appear to be utilized to a very limited extent.

**Sucrosuria.** BY HARRY SOBOTKA, MIRIAM REINER, AND S. B. WEINER. *From the Laboratories and the Pediatric Service of the Mount Sinai Hospital, New York*

Sucrose was discovered in the urine of a child 7 months old.

This sucrosuria proved of alimentary origin and reached as high a level as 2.65 per cent. Its appearance and degree depend both on the amount of sucrose given and on the nature and amount of other carbohydrates simultaneously administered. The observations were confirmed after the child had reached an age of 19 months. The mechanism of this unusual error of metabolism and the conditions of its potential experimental elicitation in normals are linked to the competitive inhibition by various sugars of the enzymatic hydrolysis of sucrose.

**Further Studies of the Enzymatic Synthesis and Hydrolysis of Cholesterol Esters in Blood Serum.** BY WARREN M. SPERRY AND V. A. STOYANOFF. *From the Chemical Laboratory, Babies Hospital, and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

Either human or dog serum catalyzes esterification of free cholesterol in serum of either species, previously inactivated by heating at 55–60° for 1 hour. Pancreatin, incubated with untreated and inactivated samples of human and dog serum with and without the addition of taurocholate, has the general effect of decreasing the ratio of combined to free cholesterol, in some experiments through inhibiting esterification and in others through catalyzing hydrolysis of cholesterol esters. The effect is greater in dog than in human serum and greater in inactivated than in untreated serum. Taurocholate activates the pancreatic enzyme or enzyme system responsible for hydrolysis of cholesterol esters. Taurocholate, added to human serum after esterification of free cholesterol has taken place, has little, if any, effect; it usually causes a relatively small and variable hydrolysis of cholesterol esters in dog serum under the same conditions. Hydrolysis of cholesterol esters in dog serum in the presence of bile salt is a rapid process, being half completed in 5 minutes and reaching a maximum within 8 hours or less. The esterification is relatively slow, approaching a maximum in 3 days. All of the findings, together with some reported previously, may be correlated with the hypothesis that the esterification of free cholesterol and the hydrolysis of cholesterol esters are in effect independent reactions catalyzed by different enzymes or enzyme systems, both of which are present in dog serum and of which only the one concerned with esterification is present in human serum.



**Ultraviolet Spectrographic Studies on Melanins.** BY MONA SPIEGEL-ADOLF.\* *From the Department of Colloid Chemistry, D. J. McCarthy Foundation, Temple University School of Medicine, Philadelphia*

In an attempt to elucidate the constitutional structure of melanins, the optical absorption power of various melanins for short wave light was studied. Irradiation products of some aromatic amino acids, so called photosynthetic melanins, tumor melanins, and sepia were used. The chemical constitution of these substances has been exactly analyzed (partly by Professor H. K. Barrenscheen). The ultraviolet spectrographic investigations show distinct quantitative differences between the photosynthetic melanins on the one hand and the genuine melanins on the other hand. While the three photosynthetic melanins show some differences among themselves, the values of their extinction coefficients are markedly lower than the extinction coefficients of the tumor melanins. In order to get comparable values, all determinations were made on solutions which contained the same amount of alkali. Because of the different alkali solubility of the various melanins, the influence of varying concentrations of alkali on the absorption coefficients of the more soluble photosynthetic melanins and of the genuine melanins was studied. Evidence was given that within a certain range of concentration the Beer-Lambert law is valid for all melanins. The differences of the extinction coefficients of the various melanins seem therefore to point to the existence of structural differences among those products. An effort was made to correlate the optical with the chemical findings.

**The Effect of Prostigmine on the Choline Esterase Activity of Human and Guinea Pig Muscle in Relation to Therapeutic Mechanism of Prostigmine in Myasthenia Gravis.** BY WILLIAM C. STADIE AND MAXWELL JONES.† *From the Laboratories of the Department of Research Medicine, University of Pennsylvania, Philadelphia*

The choline esterase activity of guinea pig muscle was deter-

\* Aided by a grant from the National Research Council, Committee on Radiation.

† Commonwealth Fellow, University of Edinburgh.

mined before and after the intravenous injection of prostigmine. No inhibition of the muscle esterase could be demonstrated, whereas that of the serum was marked. *In vitro*, the same relations were found: Only concentrations of prostigmine quite outside the therapeutic range produced inhibition of muscle esterase. In three human cases, the esterase concentration was close to that of the serum. In these cases also, therapeutic doses of prostigmine subcutaneously failed to inhibit esterase activity in the muscle. *In vitro*, experiments with human muscle gave essentially the same results as with guinea pig muscle. In a case of myasthenia gravis, the esterase activity of the muscle was within the normal range, and, *in vitro*, therapeutic concentrations of prostigmine produced no inhibition, although the activity of the serum was markedly diminished. The relation of these findings to the mechanism of the therapeutic activity of prostigmine in myasthenia gravis is discussed.

**Effect of Malonic Acid on the Reduction of Acetoacetic Acid to  $\beta$ -Hydroxybutyric Acid in Sliced and Homogenized Liver under Aerobic and Anaerobic Conditions.** BY IRENE E. STARK AND PHILIP P. COHEN. *From the Department of Physiological Chemistry, University of Wisconsin, Madison*

The reduction of acetoacetic acid to  $\beta$ -hydroxybutyric acid by liver slices is inhibited by malonic acid under aerobic conditions. Under these conditions malonic acid also inhibits the disappearance of acetoacetic acid by some mechanism other than reduction to  $\beta$ -hydroxybutyric acid. This may serve to explain the reported "ketogenic" action of malonic acid when acetoacetic acid production alone is being used as a measure of ketogenesis.

Anaerobically, liver slices show a greater reduction of acetoacetic acid to  $\beta$ -hydroxybutyric acid than aerobically. Malonic acid is without effect when anaerobic conditions obtain.

Liver tissue homogenized by the method of Potter and Elvehjem does not reduce acetoacetic acid to  $\beta$ -hydroxybutyric acid in significant amounts, either aerobically or anaerobically.

**Solubility Behavior of Crystalline Pepsin and Other Proteins.** BY JACINTO STEINHARDT. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The isoelectric solubility of crystalline pepsin (swine), as of

many other proteins, is a function of the ratio of saturating body to solvent volume. Measurements of protein nitrogen and catalytic activity in successive portions of solvent,  $\Delta V$ , equilibrated with pepsin crystals, show another aspect of this dependence. For constant  $\Delta V$  the pepsin dissolved is a constant fraction of the amount dissolved in the preceding portion, until both residue and solubility have been reduced to less than one-eighth their initial value. This relation holds at ionic strengths between 0.002 and 0.10.

This regularity, as well as relations shown by direct variation of the saturating body to volume ratio (including several of Sørensen's experiments with other proteins), is described by the equation

$$S = A \left( 1 - e^{-\frac{kV}{A}} \right) \quad (1)$$

in which  $S$  is the amount dissolved in the cumulative volume  $V$  ( $= n\Delta V$ ) and  $A$  is the total soluble quantity. Log solubility ( $\Delta S/\Delta V$ ) varies linearly with  $\Delta V$ , but the slope,  $k/A$ , and the zero volume intercept,  $\log (\Delta S/\Delta V)_0$ , are characteristic of protein and solvent, and are related more regularly to ionic strength than are the untreated data. The relation between  $\log (\Delta S/\Delta V)_0$  and ionic strength has a limiting slope close to that previously found for such dissimilar proteins as ovalbumin and hemoglobin.

The assumption that protein crystals are solid solutions in which the protein mole fraction is low, as a consequence of high molecular weight, leads to an equation, analogous to (1), in which  $k/A$  becomes  $k/R$ , where  $R$  is the quantity of insoluble impurity. Implicit in this view is the possibility, which has been confirmed experimentally, of considerably increasing the solubility of crystalline pepsin by additional fractional crystallizations. Other implications are under investigation. Whatever the interpretation, the equation describes the data, obtained in different ways, for several crystalline proteins.

**Glutathione in Relation to Growth of Rats Maintained on Diets Containing Bromobenzene and Naphthalene.** BY JAKOB A. STEKOL. *From the Department of Chemistry, Fordham University, New York*

We have shown previously that glutathione, in contrast to

cystine or methionine, does not augment the synthesis of *p*-bromophenylmercapturic or *l*- $\alpha$ -naphthalenemercapturic acids in the rat. It appeared of interest to investigate the growth-promoting power of glutathione in rats which ceased to grow because of the presence of naphthalene or bromobenzene in a diet which was adequate to induce growth of rats when fed without the hydrocarbons.

We find that 0.32 gm. of glutathione added to 100 gm. of the diet which contained 0.25 gm. of naphthalene promotes growth of rats, while this amount of glutathione is not sufficient to induce growth of rats which received 0.5 gm. of naphthalene per 100 gm. of the same diet. 1 per cent of bromobenzene in the diet and 0.32 per cent of glutathione in the diet did not, however, inhibit growth of rats. Inasmuch as the extent of the synthesis of both mercapturic acids in rats from an equivalent dose of naphthalene and bromobenzene is about the same, the relative inefficiency of glutathione in promoting growth on the naphthalene diet is noteworthy.

The results seem to indicate that, under the experimental conditions, the promotion of growth by glutathione is due to the sparing action of glutathione on that cysteine or methionine which is utilized by the rat for detoxication and growth purposes rather than to a direct participation of glutathione in the synthesis of mercapturic acids.

**The Relation of Dietary Fat to the Vitamin B<sub>1</sub> Requirement of Growing Rats.** BY F. E. STIRN AND AARON ARNOLD. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

Young rats placed on a modified vitamin B<sub>1</sub>-low ration (No. 112\*) developed polyneuritis in 4 to 6 weeks. When the polyneuritic animals were given an excess of vitamin B<sub>1</sub>, they grew 3.0 to 3.5 gm. per day and appeared normal in every way. If the basal ration was modified by replacing the 60 parts of sucrose with an isocaloric amount of fat (26 parts) and fed to the poly-

\* Ration 112 has the following composition: sucrose 62, purified casein 18, autoclaved peanuts 10, autoclaved yeast (bakers') 4, Salts I 4, factor W preparation  $\cong$  2 liver extract, 2 to 3 drops of halibut liver oil fed twice weekly to supply vitamins A and D.

neuritic rats, a similar response was obtained without the addition of any vitamin.

The following fats when introduced at a level of 40 per cent in the diet cured the polyneuritis and produced normal growth: coconut oil, butter fat, corn oil, olive oil, lard, cottonseed oil, peanut oil, synthetic tricaproin, and triacetin. The time of vaginal opening and appearance of estrus cycles were normal in the animals carried on these diets. Autoclaving of the natural oils for 10 hours did not decrease the activity. Lower levels of fat cured the polyneuritis but did not give normal growth.

The cocarboxylase and vitamin B<sub>1</sub> content of the liver and brains of the animals cured on the fat diet was very similar to that of organs from polyneuritic rats.

These results demonstrate that the faulty carbohydrate metabolism due to vitamin B<sub>1</sub> deficiency can be corrected by substituting fat for the carbohydrate in the diet.

#### **Spectrophotometric Determination of the Equilibria between Oxidation-Reduction Systems; the Potential of Cytochrome C.**

BY ELMER STOTZ, A. E. SIDWELL, JR., AND T. R. HOGNESS.

*From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago*

By means of an accurate photoelectric spectrophotometer and with the selection of two suitable wave-lengths, it is possible to determine the ratios of oxidized and reduced forms of two oxidation-reduction substances which are in equilibrium with each other. Knowing the potential of four selected reference substances (Clark's indophenol indicators), that of cytochrome was calculated from the equilibrium constants obtained. This method was first tested for accuracy by the use of two indophenol indicators of known potential, as well as by electrometric titration, and proved to be reliable.

The potential of pure cytochrome C (Keilin's procedure, from heart muscle), determined by the use of four different indicators and quinhydrone, is nearly 0.15 volt higher than reported for impure yeast cytochrome C, and does not vary with pH over the range 5.0 to 7.5.

#### **The Determination of Blood Iodine.** BY H. S. STRICKLER AND D. A. WILSON. *From the Research Laboratory of the Elizabeth*

*Steel Magee Hospital and the Department of Physiological Chemistry, School of Medicine, University of Pittsburgh, Pittsburgh*

A method employing alkali ashing is presented, by the use of which the normal blood iodine level in Pittsburgh is found to be comparable with the low normal values recently reported by Stevens\* and Baumann and Metzger,† who used chromic acid oxidation. Recoveries of iodine in each stage of the procedure are shown. The question of loss of iodine in open ashing in the presence of alkali is discussed, as well as the possible interference of traces of organic matter.

**Chemical Studies in Hypertension.** BY M. X. SULLIVAN.

*From the Chemo-Medical Research Institute, Georgetown University, Washington*

By means of new and highly specific colorimetric methods an investigation was made of the urine in hypertension for unsymmetrical dimethylguanidine, which has been considered to play a rôle in arterial hypertension. No unsymmetrical dimethylguanidine was found either normally or in hypertension. The possibility of chemical findings of value in the study of arterial hypertension is indicated.

**The Use of Milk Treated with Hydrogen Sulfide for the Development of a Copper Deficiency in the Rat.** BY WILLIAM H. SUMMERSON. *From the Department of Biochemistry, Cornell University Medical College, New York City*

The use of an exclusive diet of raw whole milk for the development of a copper deficiency in the rat is complicated by the presence of copper in the milk itself. The amounts of copper furnished by milk are variable and may be of the same order of magnitude as the animal's daily requirement. These circumstances may determine whether or not it is possible to develop a copper deficiency on a milk diet.

It has been found that milk on which a copper deficiency will ordinarily not develop may be made suitable for this purpose by preliminary treatment with hydrogen sulfide. The development of anemia on this treated milk in the presence of added iron is

\* Stevens, C. D., *J. Lab. and Clin. Med.*, **22**, 1074 (1937).

† Baumann, E. J., and Metzger, N., *J. Biol. Chem.*, **121**, 231 (1937).

more rapid and more complete than has been obtained in this laboratory on any other type of milk, including raw whole milk collected directly in glass vessels and free from copper contamination. A blood hemoglobin content of between 2 and 4 gm. per cent is routinely reached within 6 weeks from weaning, even though iron has been present in the diet throughout the experimental period.

Small amounts of added copper cure the animals, and the action of copper cannot be duplicated by lead or arsenic. The effect of hydrogen sulfide is apparently confined to the copper in the milk, since the addition of copper to the treated milk gives a diet which is indistinguishable from the untreated milk supplemented with copper in supporting growth and hemoglobin formation in the presence of added iron.

**Oxygen Uptake of Rat Tissues in Avitaminosis.** BY BARNETT SURE AND JAMES DEWITT. *From the Laboratory of Agricultural Chemistry, University of Arkansas, Fayetteville*

Records were taken with the Barcroft apparatus of oxygen consumption of several tissues of the albino rat. The experiments were carried out by the paired feeding method, the daily food intake of the control animals having been limited to that consumed by the pathological litter mates the previous 24 hours. The results, expressed as per cent reduction in oxygen uptake, are summarized as follows: Deficiency of the vitamin B complex: kidney, twenty-one groups, -15 per cent; heart, nine groups, -18 per cent. Vitamin B<sub>1</sub> deficiency associated with polyneuritis: cerebrum, sixteen groups, -16 per cent; heart, fourteen groups, -17 per cent; kidney, nineteen groups, -19 per cent. Vitamin A deficiency: cerebrum, thirteen groups, -14 per cent; heart, eight groups, -12 per cent.

Neither the extent of loss of weight, nor the severity of polyneuritis, nor the severity of ophthalmia had any influence on the quantitative reduction of oxygen uptake of either the kidney, heart, or cerebrum of the albino rat on diets deficient in vitamin B complex, vitamin B<sub>1</sub>, or vitamin A, respectively.

**The Biological Assay of the Antihemorrhagic Factor (Vitamin K).**

BY SIDNEY A. THAYER, D. W. MACCORQUODALE, R. W. Mc-

KEE, AND EDWARD A. DOISY. *From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

A curative method of assay which appears to have certain advantages over methods previously used (Dam and Schønheyder; Almquist and Stokstad) has been developed. White Leghorn chicks 1 day of age were placed on a diet free from the antihemorrhagic factor and after marked deficiency had developed were given an active extract of the vitamin on 3 successive days. On the 4th day blood was drawn and the clotting time determined. A clotting time of 10 minutes or less was considered normal.

Standardization curves were developed according to the procedure proposed by Trevan in his fundamental study of bioassay methods. For each dosage 85 to 100 birds were used in groups of seventeen to twenty at a time. The unit is defined as that quantity of material required to reduce the clotting time of the blood of 50 per cent of the chicks to 10 minutes or less. The accuracy of the method was checked by the administration of "unknowns" prepared from the extract which had been used in the preparation of the curve, groups of ten birds being used for each assay.

With the aid of this procedure extracts of alfalfa containing the antihemorrhagic vitamin have been concentrated to such a degree that preliminary assays indicate a potency of approximately 1 microgram per unit.

**The Stabilization of Color in the Determination of Histidine by the Diazo Method and Arginine by Weber's Method (Sakaguchi Reaction).** BY LLOYD E. THOMAS. *From the Biochemical Laboratory, Stanford University, California, and the Department of Biochemistry, University of Missouri School of Medicine, Columbia*

In the Hanke and Koessler diazo method for histidine determination maximum color is reached in approximately 5 to 6 minutes and fading begins in approximately 10 to 12 minutes after mixing, regardless of the order of adding the reagents.

When histidine was precipitated by mercuric salts, the precipitate dissolved in NaCN solution, and the diazo method applied by adding the  $\text{Na}_2\text{CO}_3$  to this solution, followed by the diazonium solution, the color developed as rapidly as usual and remained



stable for 30 minutes or longer. Whereas in the Hanke and Koessler method more color develops at 0° than at room temperature, in the new procedure more color develops at room temperature than at 0°.

In the Weber method for arginine determination, based on the Sakaguchi reaction, maximum color is reached in approximately 3 minutes and fading begins in 6 to 8 minutes after the last reagent is added. When arginine was precipitated by mercuric salts, the precipitate dissolved in HCl, and the Weber method applied, the maximum color was reached within 3 to 4 minutes and fading did not occur for at least 1 hour.

A Pulfrich photometer was used for color observations.

**The Influence of Optical Isomerism on the Utilization of Amino Acids by the Mouse for Growth.** BY JOHN R. TOTTER AND CLARENCE P. BERG. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

Recently Kotake, Ichihara, and Nakata\* reported that the mouse differs from the rat, presumably qualitatively, in its ability to utilize *d*-tryptophane for growth. Unfortunately the experimental data on the mice are not convincing nor are they comparable with those on the rats. Studies which we have made thus far indicate that supplements of 0.2 per cent of *d*-tryptophane added to a tryptophane-deficient basal diet allow the mouse to grow, but at a rate inferior to that induced by the same amount of *l*-tryptophane. Preliminary tests have shown that 0.5 per cent of *d*-histidine monohydrochloride usually retards the rapid loss in weight shown by control mice on a histidine-deficient diet, but does not allow growth; 0.5 per cent of *l*-histidine monohydrochloride induces good growth. As in the rat, diets deficient in lysine allow maintenance, supplements of 0.5 per cent of *d*-lysine induce growth, but 0.5 per cent of the *l* isomer is ineffective. Confirmatory studies and similar tests on other amino acids are in progress. Thus far it would appear that the differences between the growth responses of rats and mice are quantitative, rather than qualitative, in character.

\* Kotake, Y., Ichihara, K., and Nakata, H., *Z. physiol. Chem.*, **248**, 253 (1936).

**The Effect of Hepatectomy upon the Non-Protein Nitrogen Metabolism of Dalmatian Dogs.** BY HARRY C. TRIMBLE AND STEPHEN J. MADDOCK. *From the Biochemical Laboratory of Harvard Medical School and the Laboratory for Surgical Research, Boston City Hospital, Boston*

Dogs of the Dalmatian breed have been hepatectomized by means of procedures developed by Mann and his associates. The animals survived 12 to 30 hours. A study of changes in concentrations of the non-protein nitrogen fractions of the blood and of the urine has been made. The interrelation of the changes observed and their significance with respect to the survival of the animal are indicated. The fractions determined included urea, amino acids, ammonia, uric acid, allantoin (urine only), creatinine, and creatine. Together these comprise the entire nitrogen content of the urine but do not account for all non-protein nitrogen in the blood of hepatectomized animals.

**The Synthesis of Dicholylcystine and Related Substances.** BY SIDNEY F. VELICK AND JULIUS WHITE. *From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*

It is usually maintained that the taurine necessary for the biological synthesis of taurocholic acid is derived from cystine. Although oxidation prior to conjugation is generally assumed, the possibility of conjugation of cystine with cholic acid and subsequent oxidation has also been suggested. In order to study the biological aspects of this problem, we have synthesized dicholylcystine by the application of methods used in the synthesis of conjugated bile acids. The oxidation of dicholylcystine by bromine has also been studied.

**Studies on Cystamine in the Dog.** BY ROBERT W. VIRTUE AND MILDRED E. DOSTER-VIRTUE. *From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans, and the Department of Chemistry, University of Denver, Denver*

Fasting bile fistula dogs were fed 2.8 gm. of cholic acid daily. On the 3rd day of the fast an equivalent amount of cystamine dihydrochloride was administered either orally or subcutaneously

in addition to the cholic acid. Somewhat more than half the extra sulfur arising from the cystamine appeared in the sulfate fraction of the urine. The amount of taurocholic acid excreted in the bile furnished no evidence for the production of taurine from cystamine.

**A Study of the Guanidine-Like Substance in the Blood from Dogs Having Uranium Nephritis.** BY C. J. WEBER. *From the Department of Internal Medicine, University of Kansas School of Medicine, Kansas City*

Nephritis was produced in dogs by the injection of uranium nitrate (20 mg. per kilo). The tungstic acid filtrate of blood obtained from these dogs was extracted with Lloyd's reagent. The Lloyd's reagent was eluted with barium hydroxide solution. This extract contains a substance which gives a ferricyanide-nitroprusside color reaction similar to that given by a solution of glycoeyamidine.

The substance responsible for this color reaction is precipitated by phosphotungstic and picric acids. A solution containing this substance gradually increases in substances giving the Sakaguchi reaction on standing with sodium hydroxide and reaches a maximum in approximately 18 hours. This increase is similar to that given by a solution of glycoeyamidine under similar conditions. This suggests the possibility that we are dealing with a closed ring guanidine derivative similar to glycoeyamidine.

This substance may reach a concentration in nephritic blood, as calculated from its concentration in the Lloyd's extract with glycoeyamidine as a standard, of 2 mg. per 100 cc.

**Catalytic Reduction of Methemoglobin to Hemoglobin by Methylene Blue.** BY WILLIAM B. WENDEL. *From the Department of Chemistry, University of Tennessee, College of Medicine, Memphis*

Methylene blue injected in small amounts into humans or experimental animals suffering from severe methemoglobinemia due to nitrite, acetanilide, or sulfanilamide causes a prompt disappearance of the methemoglobin and an equivalent increase in oxygen capacity. The reduction of methemoglobin to hemoglobin by the dye, also observed in blood *in vitro*, is a reaction of

catalysis, indeed, a reduction catalysis. Leucomethylene blue formed by reaction of methylene blue with some cellular constituent reduces methemoglobin to hemoglobin and becomes methylene blue again. This type of catalysis, heretofore overlooked, may explain the failure of methylene blue and similar oxidation-reduction systems to increase the oxygen consumption of intact animals, most isolated tissues, and cell suspensions.

The methemoglobin-antagonizing effect of methylene blue does not negate the evidence that methylene blue converts hemoglobin to methemoglobin (*in vivo* and *in vitro*) nor the writer's explanation for the dye's antagonism towards cyanide in the intact animal.

**Loss of the Carbohydrate Metabolism Factor during the Boiling of Vegetables.** BY LAURENCE G. WESSON. *From the Veader Laboratory of Experimental Therapeutics, Baltimore*

Abnormally high respiratory quotients during carbohydrate assimilation are given by rats that have been maintained for a number of months on a fat-deficient diet. These high quotients, many of them above 1, have been attributed to a lack of an accessory factor in the fat-deficient diet that is necessary for carbohydrate metabolism.\* It has now been found that rats on a diet of boiled vegetables exhibit this abnormality after 3 months time (average maximum R.Q. = 1.05), although the vegetables in the unboiled condition contain ample amounts of the factor. This indicates that some loss of the factor occurs during the boiling of the vegetables. The question is raised as to whether a deficiency of the carbohydrate metabolism factor may not occur in the average human diet, and lead at times to pre-diabetic obesity and diabetes.

**The Destructive Action of Acidified Candies upon Tooth Enamel.**

BY EDWARD S. WEST AND FREDERICK R. JUDY. *From the Laboratory of Biochemistry, University of Oregon Medical School, Portland*

Acids of carbohydrate fermentation are considered a cause of dental caries. Hard candies are often acidified with citric acid. Strong solutions of these candies showed pH values in water of

\* Wesson, L. G., *J. Biol. Chem.*, **73**, 507 (1927) and ff.

2.5 and in saliva of 3.4. Teeth were mounted in rubber stoppers with wax to protect the roots and the stoppers fitted with bottles containing solutions of candies in water and in saliva. The bottles were agitated at room temperature for 24 hours and the calcium and phosphorus in the solutions determined. A 40 per cent solution of lime drops in saliva, pH 3.5, dissolved 10 mg. of enamel mineral. A chalky residue on the surface of teeth so treated had a Ca:P ratio of 2.9 (enamel mineral 1.67), indicating an ionic exchange of phosphate and citrate ions in the enamel surface. This was supported by equilibrating tricalcium phosphate with citrate buffers and determining Ca:P ratios in the solid and liquid phases. 50 cc. of 0.1 M citrate buffer, pH 3.88, were equilibrated with 0.5 gm. of tricalcium phosphate for 8 days. The Ca:P ratio in the solid phase was 5.4 and in the liquid phase 0.31, indicating a citrate-phosphate ionic exchange in the surface of the solid.

Citric acid candies cause caries by solution of tooth mineral and by erosion due to ionic exchange. The work is being continued.

**The Ketonic Estrogen of Sow Ovaries.** BY W. W. WESTERFELD, D. W. MACCORQUODALE, SIDNEY A. THAYER, AND EDWARD A. DOISY. *From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis*

Sow ovaries were extracted with hot ethyl alcohol, the extract purified, and the estrogens separated into a ketonic and a non-ketonic fraction by means of Girard's reagent. That the activity of the ketonic fraction was due to a ketonic estrogen was demonstrated by inactivation of this fraction with semicarbazide. Moreover, a second treatment of this fraction with Girard's reagent resulted in a recovery of all of the estrogen in the ketonic fraction.

This ketonic estrogen was identified as theelin by the following comparisons of the ketonic fraction with pure theelin. (1) Reduction with sodium and alcohol increased the activity 400 to 800 per cent, and with a platinum catalyst, 800 per cent; this is identical with the increases in activity given by theelin similarly reduced. (2) The partition ratio of the ketonic fraction between benzene and 70 per cent ethyl alcohol was 3:1, which was identical with the ratio obtained for theelin. (3) The partition ratio of the

product obtained by reduction of the ketonic fraction was 1:1, which was identical with the ratio obtained for dihydrotheelin. (4) According to the methods of assay employed, the weight of 1 rat unit of the ketonic fraction was found to be equivalent to the weight of 15 mouse units; the rat unit to mouse unit ratio for theelin was 1:13. (5) The weight of 1 rat unit of the product obtained by reduction of the ketonic fraction was equivalent to the weight of 3 to 4 mouse units; the rat unit to mouse unit ratio for dihydrotheelin was 1:3.

Our conclusion is that the ketonic estrogen in sow ovaries is principally, or perhaps entirely, theelin.

**The Metabolism of  $\beta$ -Naphthylamine.** BY FRANK H. WILEY.

*From the Haskell Laboratory of Industrial Toxicology, Wilmington, Delaware*

The production of bladder tumors after the ingestion of commercial  $\beta$ -naphthylamine\* presents a unique problem in carcinogenesis in that the tumors are produced at the site of excretion rather than at the site of entrance of the causative agent. Since it is entirely possible that the absorbed material is not carcinogenic in itself, but that the carcinogenic agent is formed from it in the body, a study of the nature of the metabolites of  $\beta$ -naphthylamine was undertaken in order that their activity in this respect might be determined.

A preliminary study of the sulfur distribution in the urine of dogs, before and after the administration of  $\beta$ -naphthylamine, indicated that a large part of this material was probably excreted as an organic sulfate and a small portion might be present in the form of a mercapturic acid derivative.

Several hundred mg. of a compound melting at 224° have been isolated from the urine of dogs receiving 300 mg. of  $\beta$ -naphthylamine daily. The analysis of this material gave the following results: C 50.55, H 3.71, N 5.54, S 13.14; theoretical for  $C_{10}H_9NSO_4$ , C 50.21, H 3.77, N 5.86, S 13.39, respectively.

The action of chlorosulfonic acid on 2-amino-1-naphthol in a mixture of carbon disulfide and dimethylaniline gave a compound melting at 222°. Both the isolated and synthetic compound on

\* Hueper, W. C., Wiley, F. H., and Wolfe, H. D., *J. Ind. Hyg. and Tox.*, 20, 46 (1938).

hydrolysis with N sulfuric acid and treatment with ammonia gave the purple ether-soluble pigment described by von Auwers\* and which he found to be produced on oxidation of 2-amino-1-naphthol by atmospheric oxygen in the presence of ammonia. It seems evident from these data that the material isolated from the urine of these animals was the acid sulfate of 2-amino-1-naphthol; attempts to isolate the mercapturic acid derivative of 2-amino-1-naphthol have thus far been unsuccessful.

**The Chemistry of Human Epidermis. IV. The Effect of Certain Salts upon the  $\zeta$ -Potential of the Stratum Corneum.** BY VERNON A. WILKERSON. *From the Department of Biochemistry, Howard University Medical School, Washington*

The electrophoretic velocity of particles of human skin was measured in a modified Northrop-Kunitz apparatus, with a flat microelectrophoresis cell. The following salts were used in dilution of 0.1 M to 0.00002 M: Series I, chlorides of sodium, potassium, and lithium; Series II, sodium salts of chlorine, bromine, and iodine; Series III, chlorides of calcium, magnesium, and barium; Series IV, aluminum chloride and ferric chloride.

The  $\zeta$ -potential-concentration curves were determined. The salts of Series I and II in dilute solutions enhanced the negativity of the particles, causing a high maximum in the curves. Increased concentrations diminished the charges to approximately zero, with no reversal of the sign. Specific ionic effects were exhibited by the alkali halides having a common negative ion,  $\zeta$  decreasing,  $\text{Na} > \text{K} > \text{Li}$ ; for salts with varying negative ions the order  $\text{Cl} > \text{I} > \text{Br}$  was observed. Series III, divalent cations with a common univalent anion, exhibited in dilute solutions slight increases in the negativity of the particles. Increased concentrations did not reverse the sign of the charge. Series IV, trivalent cations with a common anion, showed a rapid reversal of the sign of the charge to a positive maximum, which was followed by an increase of negativity approaching zero potential.

In comparing the specific ionic effects of the halides on the human skin with that obtained on inert surfaces it is apparent that among the cations there is a preferential adsorption of sodium, while among the anions the same appears to be true of chlorine.

\* von Auwers, K., *Fortschr. chem. Physik u. physik. Chem.*, **18**, 45 (1924).

**Mechanism of the Globin-Ferrihemate Conjugation.** By EDWARD F. WILLIAMS, JR., AND DEMPSIE B. MORRISON. *From the Department of Chemistry, College of Medicine, University of Tennessee, Memphis*

Discontinuous titration with standard hydrochloric acid of oxyhemoglobin and methemoglobin solutions of various species has been carried out and the amount of ferrihemic acid liberated at different pH values measured. The methods used have been described.\*

The titration curves break sharply at about pH 4, and simultaneously most of the ferrihemic acid is liberated. A small amount of ferrihemic acid is liberated on either side of this zone. Results with oxyhemoglobin are essentially the same as with methemoglobin, since within this pH range oxyhemoglobin is very rapidly converted to methemoglobin.

Uniformity of results indicates that the effect of hydrochloric acid is primarily on the ferrihemic acid constituent of the conjugate, since the buffer capacities of the hemoglobins of the species used differ. Morrison and Williams, in an accompanying abstract, have shown that ferrihemic acid behaves as a dibasic acid which is very insoluble at pH 4 or lower, probably because of repression of its ionization. We suggest, therefore, that the basic groups of globin may conjugate with the iron of ferrihemic acid only when the latter is ionized and exists as a negative ferrihemate radical. When the reaction of a methemoglobin solution is adjusted to about pH 4, the ionization of the ferrihemic acid is so repressed that its solubility is practically abolished (although remaining in the colloidal state), and the basic groups of the globin released. Thus we would account for the abrupt increase in buffer capacity and sudden liberation of ferrihemic acid at this critical pH.

**Urinary Estrogens.** By D. A. WILSON, H. S. STRICKLER, AND WILLIAM S. McELLROY. *From the Research Laboratory of the Elizabeth Steel Magee Hospital and the Department of Physiological Chemistry, School of Medicine, University of Pittsburgh, Pittsburgh*

A modified bioassay for urinary estrogens is described. The

\* Williams, E. F., and Morrison, D. B., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 119, p. cv (1937).



method is simple and especially adapted for clinical use in laboratories with limited facilities. Essential points are (1) a simplification of the Koch-Gallagher extractor, (2) chemical separation of estrogens from interfering androgens, and (3) proper injection and rotation of the spayed animals.

When this method and a standardized colony of full Wistar strain rats were employed, the daily 24 hour excretion of estrogens was determined for a period of 30 to 40 consecutive days for a representative number of normal females in every decade of life from 10 to 50 years of age. The results obtained in the age group of 20 years are reported graphically, as international units of estrogenic material with limits of error against consecutive days of the menstrual cycle. Preliminary results in other age groups are discussed.

**The Fate of Intravenously Injected Potassium Salts.** BY ALEXANDER W. WINKLER AND PAUL K. SMITH. *From the Department of Internal Medicine and the Department of Pharmacology and Toxicology, Yale University School of Medicine, New Haven*

Isotonic potassium bromide was injected intravenously into dogs, and simultaneous specimens of blood and urine obtained before and at intervals after injection. The concentration of bromide in the serum rose much more than that of potassium. On the assumption that these ions were uniformly distributed in the body water, their respective volumes of distribution were calculated by the formula

$$\text{Apparent volume of distribution} = \frac{\text{amount given minus amount excreted}}{\text{change in concentration in serum water}}$$

The apparent volume of distribution of bromide corresponded to about 25 per cent of the body weight, that of potassium to between 50 and 70 per cent of the body weight. These percentages correspond approximately to those of the extracellular fluid and of the total body fluid respectively, as determined by other means.

Similar studies were made after the intravenous injection of potassium chloride. The apparent volume of distribution of the potassium again corresponded to between 50 and 70 per cent of

the body weight. The concentration of chloride in the serum did not change measurably.

The experiments support the hypothesis that injected potassium, unlike bromide and chloride, diffuses freely into the cell water as well as into the extracellular fluid.

**Activity of Pyridine Derivatives in the Cure of Canine Black Tongue.** BY D. W. WOOLLEY, F. M. STRONG, AND ROBERT J. MADDEN. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

Since it has been shown that nicotinic acid and nicotinic acid amide are active in the cure of black tongue in dogs, it was of interest to investigate the anti-black tongue potency of various related compounds. A number of pyridine derivatives were accordingly tested for their ability to cure dogs suffering from black tongue. Each compound tested was fed at a level equivalent on a molar basis to at least the active dose of nicotinic acid.

Picolinic acid, isonicotinic acid, nipecotic acid, nicotinic acid N-diethylamide, quinolinic acid, 6-methylnicotinic acid, trigonelline, pyridine, and 1-methylnicotinic acid amide chloride were inactive. Nicotinic acid, nicotinic acid amide, and ethyl nicotinate possessed about the same potency and  $\beta$ -picoline showed definite activity. Nicotinic acid N-methylamide gave slight activity.

These results indicate the extreme specificity of structure necessary for anti-black tongue activity. Substitution of alkyl groups in the ring on either nitrogen or carbon, or on the amide N renders the vitamin inactive. The results also indicate that whereas the dog can oxidize a methyl group on the pyridine ring to a carboxyl, it cannot remove the labile  $\alpha$ -carboxyl in quinolinic acid by decarboxylation, nor can it hydrolyze an N-diethylamide to the acid.

**The Protein of the Casing of Salmon Eggs.** BY E. GORDON YOUNG AND W. ROBERT INMAN. *From the Department of Biochemistry, Dalhousie University, Halifax, Canada*

The protein of the egg casings of *Salmo salar* has been prepared and analyzed. It has been found to be insoluble in all ordinary solvents and slowly hydrolyzed by pepsin. The content of certain

amino acids has been determined as follows: cystine 1.84 per cent, tryptophane 1.42 per cent, tyrosine 5.12 per cent, histidine 1.26 per cent, lysine 3.51 per cent, arginine 5.79 per cent. The ratio of histidine to lysine to arginine is as 1:3:4. The protein has been classed as a pseudokeratin.

**Quantitative Studies of Carnosine and Anserine in Mammalian Muscle.** BY JOHN A. ZAPP, JR. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

Carnosine and anserine were determined in muscle by a modification of the method of Wilson and Wolff.\* Ground muscle was extracted with water, proteins were precipitated with boiling 95 per cent alcohol at pH 5, and the filtrate treated with mercuric acetate. The mercury salts were decomposed with hydrogen sulfide, and the centrifugate and washings were concentrated. Carnosine was then determined by the Koessler and Hanke diazo procedure, and the  $\alpha$ -amino nitrogen both before and after hydrolysis, by the Van Slyke method. The increase in amino nitrogen is due almost entirely to carnosine and anserine. With carnosine known from the diazo value, anserine can be calculated.

Carnosine and anserine were determined in various muscles of the cat, rabbit, dog, horse, white rat, giraffe, ox, and lion. The maximum carnosine in skeletal muscle (4.63 mg. per gm.) was found in the horse (gluteus) and the maximum anserine (5.02 mg. per gm.) in the rabbit (gastrocnemius). The minimum carnosine in skeletal muscle (0.14 mg. per gm.) was found in the ox (buccinator), and the minimum anserine (0.07 mg. per gm.) in the dog (gastrocnemius). There was considerable variation in the carnosine and anserine content of different muscles of the same species. Cardiac muscle of the ox was very low in carnosine (0.09 mg. per gm.) and contained no demonstrable amount of anserine.

The effect of feeding a liberal meat diet, and of fasting, was studied in three muscles of the dog.

\* Wilson, D. W., and Wolff, W. A. *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 100, p. cvi (1933).





